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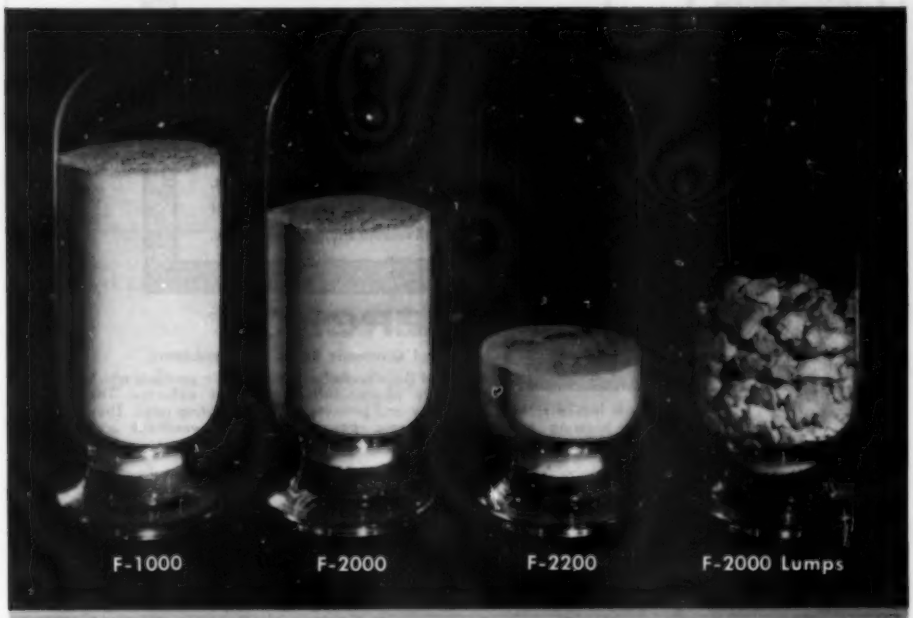

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Urinary Excretion Kinetics for Evaluation of Drug Absorption I*

Solution Rate Limited and Nonsolution Rate Limited Absorption of Aspirin and Benzyl Penicillin; Absorption Rate of Sulfaethylthiadiazole

By EINO NELSON and INGE SCHALDEMOSE

The use of urinary excretion data to evaluate drug absorption was discussed from the theoretical viewpoint and results of studies conducted to determine absorption of aspirin, benzyl penicillin, and sulfaethylthiadiazole were interpreted according to these considerations. It was shown that the absorption of aspirin was apparently rate limited by the time necessary for this drug to dissolve *in vivo* and that benzyl penicillin absorption appeared to be rate limited by the intrinsic solution rate properties of the salts used. The absorption rate of sulfaethylthiadiazole was calculated by means of excretion rate data and the results obtained shown to be in excellent agreement with calculation of the same quantity made from previously reported blood level data. The calculated absorption rate of this drug was about 1,200 mg./hr., fifteen to thirty minutes after the ingestion of 1-Gm. doses.

DRUG ABSORPTION STUDIES are usually conducted in the whole animal by measuring level of drug in blood or other fluids of distribution at various times after drug administration. This procedure does provide a direct basis for interpretations of the effect of variables introduced into experiments, but in some cases cannot be applied because small doses of drug result in less than assayable levels. In other cases, a vigorous and systematic sampling scheme may make it difficult to obtain test-subject participation. In still other cases, the information desired is of a comparative nature and direct determinations of blood levels are unnecessary. Urinary excretion data are frequently capable of supplying quantitative information on the absorption of drugs without the inconvenience of blood sampling even though these determinations are less direct. Since drug appearing in the urine may be considered concentrated therein as compared to the drug in circulation in the body fluids, then excretion data may be the only way in which the absorption of some drugs can be studied.

Information on urinary excretion has been used previously to evaluate the absorption of riboflavin (1, 2), sodium paraminosalicylate (3), chloramphenicol esters (4), aspirin, creatinine, and amphetamine on ion exchange resins (5), amphetamine in sustained release pellets (6), and tetracycline (7). This report reviews and discusses the theoretical bases for application of excretion data to evaluation of drug absorption, as well as the limitation of this application, and applies the concepts to show how *in vivo* solution rate rate-limits the absorption of aspirin and benzyl penicillin after oral ingestion, and to calculate the absorption rate of sulfaethylthiadiazole.

THEORY

Urinary excretion kinetics are one part of the broader field of the kinetics of drug absorption, distribution, metabolism, and excretion and a substantial amount of information is available in the litera-

* Received February 23, 1959, from the School of Pharmacy, University of California, San Francisco 22.
Supported in part by a Grant-in-Aid from E. R. Squibb and Sons, Division of Olin Mathieson Chemical Corp.

ture in this general field (8–26). The usefulness of urinary excretion measurements lies in the direct proportionality which exists between excretion rate and amount or concentration of drug in the blood stream.[1] When drug in blood is in equilibrium with the other fluids of distribution, then excretion rate is proportional to the concentration or amount in all fluids of distribution. The proportionality may be expressed as (6)

$$\frac{dAe}{dt} = KfA_b \qquad \text{(Eq. 1)}$$

where dAe/dt is excretion rate in amount per unit time, K is the rate constant for removal from the body in reciprocal time, f is the fraction of administered drug excreted unchanged in urine, and A_b is the amount of drug in the blood or in the body, depending on whether or not blood and other fluids of distribution are at equilibrium. Equation 1 may be written as well in terms of concentration in the blood since A_b is equal to the product of blood concentration and distribution volume, C_b and V_d, respectively. At equilibrium between blood and other fluids of distribution, V_d represents total volume of distribution. Equation 1 written in this form was the basis for determination of volume of distribution by simultaneous measurement of excretion rate and blood level (15). In any application of Eq. 1, it is implicitly understood that no changes are made in test conditions that would cause a change in renal clearance of the compound under study.

Equation 1 is equally valid when applied to the excretion of a metabolite of a drug. For example, if drug A is administered and it is transformed partially or entirely to a metabolite B, then Eq. 1 may be used to plot the amount *vs.* time course of the metabolite in the blood stream. This would, of course, require information on the value of the constants K and f and further, assurance that the site of the metabolic conversion is such that the metabolite enters the general circulation. If the rate of the conversion A to B is known, then, conceivably, excretion data on the metabolite could be used to elucidate the amount *vs.* time course of its precursor A in circulation. This would probably not be practical in most cases due to difficulties of measurement of the necessary quantities.

The application of Eq. 1 to evaluation of drug absorption is particularly direct when the information desired is of a comparative value. For example, if the absorption of a given drug in several salt forms is desired to be compared, then the ratio of excretion rates, R,

$$R = \frac{(A_b)_1}{(A_b)_2} \qquad \text{(Eq. 2)}$$

is the ratio of amounts of drug from forms 1 and 2 in the blood. Since the same drug is under consideration, the constants K and f are equal and cancel when the ratio is taken (6).

Application of Eq. 1 in the manner just described is also possible when the excretion of a metabolite is followed. The quantity R, of course, then refers to the ratio of the amounts of metabolite in circulation.

When the constants K and f are known in Eq. 1, then the actual amount in circulation may be calculated. With information as to the distribution volume of the drug, this information is reducible to concentration in the fluids of distribution. The limitation in this application lies in finding the excretion rate over relatively short periods of time. This information is probably most easily obtained by graphical determination of the slope of a plot of cumulative amount of drug excreted with time. The accuracy of the values so obtained depend both on the carefulness with which the graphical constructions are performed as well as the accuracy and precision of the assay conducted to determine urine concentration.

Equation 1 has been used as the basis for development of an expression to calculate absorption rate in terms of amount per unit time (27). The expression had the following form

$$\text{Absorption Rate} = \frac{1}{f}\left(\frac{dA_e}{dt} + \frac{d^2A_e}{Kdt^2}\right) \qquad \text{(Eq. 3)}$$

The second derivative term may be found graphically from a plot of excretion rate *vs.* time. The determination of K and f and the limitations in application of Eq. 3 have been discussed (27).

Additional theoretical considerations on interpretation of excretion data have been discussed (6, 7, 27).

EXPERIMENTAL

Conduction of Tests.—All absorption tests were conducted using adult humans in apparent good health. All drugs were ingested orally on fasting stomachs in the morning. No food was taken by subjects until at least one and one-half hours after ingestion of drug. Urine blanks were obtained immediately before drug ingestion. Five to seven subjects were used in tests depending on the drug under study, and their participation in a given test as well as their ages and weights are indicated on the tabulated data which follow later in this report. These data also indicate the urine sample collection times with the several drugs studied.

Preparation of Dosage Forms.—*Aspirin.*—Tablets of aspirin containing 325 ± 5 mg. drug were prepared from a standard precompressed granulation of aspirin containing 10% starch.[2] The test set of tablets was compressed at 5,000 p. s. i. These tablets were $^3/_8$ inch in diameter and made with standard cup punches in a Carver press modified for tablet compression. Two tablets of the set constituted a dose in the absorption studies. In addition, 650 ± 5 mg. aspirin of U. S. P. XV quality was used in the tests after dissolving this amount in about 250 ml. water. After this dose in solution was administered, the container was rinsed at least once and the rinsings also ingested.

Sulfaethylthiadiazole.—This drug was taken from a sample of commercial purity[3] and 1,000 ± 25 mg. constituted a dose. The drug was ingested after suspension in about 250 ml. of water.

Benzyl Penicillin Salts.—The potassium and procaine salts of this drug were of a grade suitable for parenteral use. A 62.5 ± 2.5-mg. dose of the po-

[1] Exceptions exist in cases where there is a threshold level such as with glucose and in cases where the body level is at such a high value as to saturate function. The latter case would ordinarily not occur with the usual range of drug doses.

[2] Merck and Co., Inc., Rahway, N. J.

[3] Kindly supplied by Dr. Stanton Hardy, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

tassium salt and 100 ± 5 mg.-dose of the procaine salt were used in the absorption study. These were administered as thin, flat-faced pellets 0.95 cm. in diameter, contained in 000 hard gelatin capsules with about 50 mg. sodium bicarbonate in each end. Pellets were chosen as to weight so that one pellet constituted a dose. The pellets were prepared by a compression technique previously described (7). No binders, disintegrating agents, diluents, or lubricants were incorporated before compression. The pellets were hard and homogenous and resisted disintegration in water for several hours (the procaine salt—the potassium salt dissolved rapidly from its exposed faces). The administration of benzyl penicillin in the form described insured that tests were conducted under conditions of essentially equal and constant surface.

Analytical Methods.—Aspirin was determined as salicylic and salicyluric acids colorimetrically using a ferric nitrate reagent (28). Benzyl penicillin was determined by cylinder-plate assay, using *Staph. aureus* 209 P as the test organism (29).[4] Sulfaethylthiadazole was determined colorimetrically by the Bratton and Marshall method. All absorbance determinations were made with a Bausch and Lomb Spectronic 20 colorimeter. Blank urine samples were assayed and appropriate corrections made when necessary.

RESULTS AND DISCUSSIONS

Aspirin Absorption in Solution and Tablets.—Excretion data from aspirin ingestion in tablets and in solution are shown in Tables Ia and Ib. The nature of the cumulative amounts excreted *vs.* time curves is shown on Fig. 1. All data were in terms of apparent amount of salicylate excreted since the assay method determines both unchanged salicylic and salicyluric acids (30). Since there was no reason to believe that the excretion ratio of these two substances changed in a given subject from test to test the comparison of amounts of apparent salicylate excreted in the two cases was a valid basis for comparison of aspirin absorption from the two dosage forms. Aspirin anion is very rapidly hydrolyzed to salicylate anion in the plasma (31), so this occurrence did not affect the comparison of absorption from the two dosage forms.

It has been calculated that the time needed to effect solution of aspirin at absorption sites in the gastrointestinal tract should rate limit absorption of this drug (32). The theoretical calculation was based on the area of absorbing surface expected to be available in the gastrointestinal tract, diffusion coefficient of aspirin, and other pertinent quantities involved (32). In the present work, the maximum mean excretion rates of salicylate were greater from aspirin in solution (Table Ic) than they were from aspirin in rapidly disintegrating tablets at comparable times. As a consequence of Eq. 1, the salicylate level in blood was greater from the solution dosage also. The present work, then supports the theoretical calculation. Data in the literature on salicylate levels in blood from dosage in solution and in tablets give additional support to the theoretical calculation (33). With regard to the data of Table I, the t-test showed that the difference in amounts excreted in the two dosage forms was significant at four hours at the 99% confidence level.

The rate-limiting nature of the *in vivo* solution step should be considered in any assessment of drug absorption when the change in experiment is concerned with change in dosage form or salt form of a particular drug (34, 35). Recognition of the importance of this factor should in some instances eliminate the necessity for experiment, or at least allow its conduction with an awareness of the possible effect of an important variable.

Benzyl Penicillin Absorption from the Potassium and Procaine Salts.—Excretion data from tests with these salts are listed in Tables IIa and IIb. The mean cumulative excretion curves are shown on Fig. 2. The differences shown were expected since the method of dosage form preparation insured that the rate limiting factor in absorption would be the intrinsic solution rate properties of each salt. These results were completely in accord with results obtained from blood level studies (42) but interpretation of the present results were less ambiguous, since

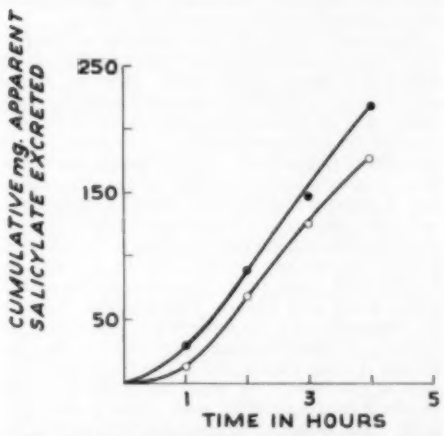

Fig. 1.—Mean cumulative amounts of apparent salicylate excreted to various times after ingestion of 0.65 Gm. aspirin in solution and in rapidly disintegrating tablets. Solid points, drug in solution; open points, drug in tablets.

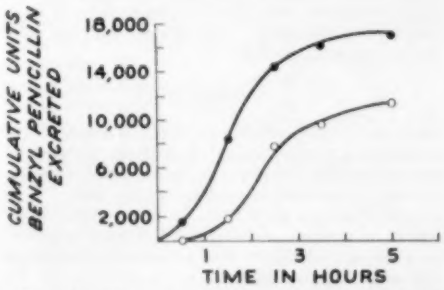

Fig. 2.—Mean cumulative amounts of benzyl penicillin excreted to various times after ingestion of 100,000-unit doses of the potassium and procaine salts in the form of limited and constant surface area pellets. Solid points, potassium salt; open points, procaine salt.

[4] The assistance of Mrs. Yoko Yuzuriha is gratefully acknowledged.

TABLE Ia.—CUMULATIVE MG. SALICYLATE AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS FOLLOWING INGESTION OF 0.65 GM. ASPIRIN IN SOLUTION[a]

Subject[b]	Excreted, mg. 1.0	2.0	3.0	4.0	Urine, ml. 1.0	2.0	3.0	4.0
O (28-68)	25.5	85.0	139.0	189.0	40	360	550	590
M (28-68)	27.5	81.0	149.0	213.0	300	650	960	1,060
N (21-77)	29.6	88.8	147.0	216.0	150	480	770	900
X (22-58)	20.6	65.0	111.9	223.5	310	490	650	830
P (26-75)	45.6	123.4	189.6	251.2	120	220	270	310
Mean	29.8	88.6	147.3	218.4	184	440	640	738

[a] Apparent salicylate—see text. [b] Bracketed quantities are subject's age in years followed by his weight in kilograms.

TABLE Ib.—CUMULATIVE MG. SALICYLATE AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS FOLLOWING INGESTION OF 0.65 GM. ASPIRIN IN TABLETS[a,b]

Subject	Excreted, mg. 1.0	2.0	3.0	4.0	Urine, ml. 1.0	2.0	3.0	4.0
O	21.8	69.5	178.5	182.0	80	440	510	560
M	0.6	60.1	118.7	167.1	40	190	330	390
N	18.4	59.2	107.6	166.9	220	340	380	660
X	21.0	82.5	144.5	196.5	180	380	540	700
P	19.2	72.5	130.0	176.5	70	110	150	190
Mean	12.4	68.8	125.9	177.8	118	292	582	500

[a] Apparent salicylate—see text. [b] Mean disintegration of eight by U. S. P. XV method equal to four seconds.

TABLE Ic.—EXCRETION RATES OF APPARENT SALICYLATE AFTER INGESTION OF 0.65 GM. ASPIRIN IN SOLUTION AND IN RAPIDLY DISINTEGRATING TABLETS[a,b]

Time, hr.	Excretion Rate, mg./hr. Solution	Tablets
0.5	30	10
1.0	45	35
1.5	58	42
2.0	75	65
2.5	70	57
3.0	62	50
3.5	57	45

[a] Computed from data on Fig. 1 by graphical determination of slopes at indicated times.
[b] Values shown determined over half-hour intervals having indicated times at their midpoint.

it could be argued that the effects observed in the cited work might be due to differences in drug release properties of the dosage form.

It has been shown previously that the salts of the same acid may dissolve at widely different rates (34–36). The rapid *in vivo* solution rate of the potassium salt of benzyl penicillin was due to the properties of the diffusion layer that surrounds this material (or other potassium or sodium salts of weak acids) as it dissolved. The Noyes–Whitney solution rate law states that

$$\frac{da}{dt} = KS(C_s - C) \qquad \text{(Eq. 4)}$$

where da/dt is the solution rate, K a constant, S the surface area, C_s the concentration of a saturated solution of the dissolving species, and C the concentration in the dissolution medium at any time. If *in vivo* solution took place in the stomach when the pellets of potassium benzyl penicillin were ingested, then Eq. 4 could be written to describe dissolution therein by substituting for C_s as

$$\frac{da}{dt} = KS(C_o(1 + K_a/[H^+]_d) - C) \qquad \text{(Eq. 5)}$$

where C_o is the solubility of the undissociated acid (37), K_a the acid's dissociation constant, and $[H^+]_d$ the hydrogen ion concentration of the diffusion layer. This latter quantity would be the hydrogen ion concentration of a saturated solution of potassium benzyl penicillin in gastric fluid which would be essentially that of a saturated solution of this salt in water, since preparation of the saturated solution would necessitate adding the salt without limit to either fluid and in the case of gastric fluid, its acid content would be neutralized. In acidic gastric fluid, C would be essentially equal to C_o and Eq. 5 reduces to

$$\frac{da}{dt} = KSC_o K_a/[H^+]_d \qquad \text{(Eq. 6)}$$

Equation 6 should also hold in more alkaline regions of the gastrointestinal tract since absorption constantly decreases drug titer in the fluids there and this process should allow equation of C to C_o.

Equation 6 should also be applicable to description of the *in vivo* dissolution of the procaine salt of benzyl penicillin in gastric fluid. However, in the case of this salt the diffusion layer pH would be much nearer that of the medium, since it would be represented by a saturated solution of procaine benzyl penicillin in gastric fluid. At equilibrium this would be the hydrogen ion concentration of a saturated solution of procaine hydrochloride, benzyl penicillin free acid, and some anion. It seems reasonable to expect that the hydrogen ion concentration in the diffusion layer in the two cases would differ by at least one hundred-fold, hence the *in vivo* solution rates should differ by the same factor. This difference must be the explanation for the difference in absorption, as reflected in excretion rates observed soon after ingestion of drugs (Table IIc). The degree of difference in excretion rates would no doubt be much greater if benzyl penicillin were stable in acid medium. The pellets of its potassium salt could be expected to have dissolved completely in the stomach and this was probably not the case with the procaine salt because of its slower solution rate. Substantial amounts of penicillin from the potassium salt would be degraded since the half-life of benzyl penicillin at pH 2 and 37° is of the order of ten minutes (38).

TABLE IIa.—CUMULATIVE UNITS BENZYL PENICILLIN AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS AFTER INGESTION OF 100,000 UNITS OF POTASSIUM SALT[a,b]

Subject[c]	Units Excreted					Urine, ml.				
	0.5	1.5	2.5	3.5	5.0	0.5	1.5	2.5	3.5	5.0
M (28-63)	0	8,600	20,800	24,300	26,300	20	160	450	640	910
E (39-77)	3,100	10,100	13,200	13,700	14,000	60	110	180	250	420
S (33-57)	2,700	6,700	9,000	9,700	10,200	40	70	170	300	420
P (26-75)	900	8,200	10,200	10,800	11,600	40	120	240	310	340
I(33-61)	700	8,700	18,900	22,400	23,600	30	80	120	150	230
Mean	1,480	8,460	14,420	16,180	17,140	38	108	232	330	464

[a] Dose contained in a single pellet 0.95 cm. in diameter—see text.
[b] Shown to the nearest 100 units.
[c] Bracketed quantities are subject's age in years followed by his weight in kilograms.

TABLE IIb.—CUMULATIVE UNITS BENZYL PENICILLIN AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS AFTER INGESTION OF 100,000 UNITS OF PROCAINE SALT[a,b]

Subject	Units Excreted					Urine, ml.				
	0.5	1.5	2.5	3.5	5.0	0.5	1.5	2.5	3.5	5.0
M	0	2,400	7,900	10,100	14,800	20	80	150	250	430
E	0	1 300	2,900	3,200	3,400	30	340	550	620	690
S	0	1,600	4,400	8,200	10,900	10	60	230	520	810
P	0	1,600	2,700	3,400	..	20	100	180	210	..
I	0	2,900	21,200	23,200	24,500	20	110	270	310	410
Mean	0	1,960	7,820	9,600	13,400	20	138	276	382	585

[a] Dose contained in a single pellet 0.95 cm. in diameter—see text. [b] Shown to the nearest 100 units.

TABLE IIc.—EXCRETION RATES OF BENZYL PENICILLIN AFTER INGESTION OF 100,000 UNITS OF THE POTASSIUM AND PROCAINE SALTS IN THE FORM OF LIMITED AND CONSTANT SURFACE AREA PELLETS[a]

Time, hr.	Excretion Rate, Units/hr.	
	Potassium salt	Procaine Salt
0.5	4,000	400
1.0	6,800	1,600
1.5	8,800	3,600
2.6	6,000	6,400
2.5	3,600	4,600
4.0	800	1,200

[a] Mean rates computed from curves on Fig. 2 by graphical determination of slopes at indicated times.

Differences in absorption rate have been noted many times when blood levels from administration of slightly soluble weak acids as the acid or slightly soluble salt have been compared to the blood levels obtained from the administration of the same acid as the potassium or sodium salt. The results obtained in the present work with benzyl penicillin are in accord with other studies using the same compounds (42). Higher blood levels were obtained from administration of the sodium and potassium salts of para-aminosalicylic acid as compared to the free acid (43), from the sodium salts of sulfapyridine and sulfathiazole as compared to their respective free acids (44), from the sodium salt of novobiocin as compared to the free acid (45), from the potassium salt of phenoxymethyl penicillin as compared to the free acid (46), and it has also been observed that hippuric acid was excreted at a more rapid rate when sodium benzoate was ingested as compared to the excretion rate when benzoic acid was ingested (47). These observations along with the present work validate the *in vivo* solution rate explanation. Other considerations on this topic were discussed (35).

The comparative absorption kinetics in the case of the salts of benzyl penicillin are easily interpreted with the aid of Eq. 2 from data in Table IIc. The average blood level ratios, potassium to procaine salt, at various times are listed below:

Time, hr.	R
0.5	10.0
1.0	4.2
1.5	2.4
2.0	0.93
2.5	0.78
4.0	0.67

The literature (39) on penicillin distribution and excretion in a normal human contains information that allows calculation of the constants K and f in Eq. 1. The half-life of benzyl penicillin in circulation was about 0.46 hours which allows assignment of 1.5 hours^{-1} as the value of the constant K. Further, it appeared that about 80% of injected penicillin was excreted as unchanged material, allowing the assignment of 0.8 for the value of f. These values allow writing Eq. 1 as $da_e/dt = 1.2\,A_b$. Since the maximum excretion rate from the potassium salt was 8,800 units/hour, the maximum amount of penicillin in circulation was about 7,300 units. The penicillin data (39) may be interpreted in terms of a relationship derived previously (15) to determine a penicillin volume of distribution of about 19 L. In the present case, then, the maximum concentration from the potassium salt was about 0.38 units/ml. This value was about one-half of the reported maximum blood levels after 200,000-unit oral doses of potassium benzyl penicillin in capsules in humans (40). The bases for these calculations were from limited data and were only included to illustrate full application of Eq. 1.

Absorption Rate of Sulfaethylthiadiazole.—The tabulated data of Table III show the amounts of this drug excreted to various times. These data were used to calculate its absorption rate by Eq. 3 since data were available in the literature to calculate absorption rate for this drug from blood level measurements (41). An agreement between calculated values would tend to show the essential soundness of the excretion rate approach to study drug absorption.

The means of the cumulative amounts excreted data shown in Table III are plotted against time on

TABLE III.—CUMULATIVE MG. SULFAETHYLTHIADIAZOLE AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS AFTER INGESTION OF 1.0 GM. DRUG IN SUSPENSION[a]

Subject[b]	Drug, mg. 0.5	1.5	2.5	3.5	6.0	8.0	Urine, ml. 0.5	1.5	2.5	3.5	6.0	8.0
O (28-68)	5.4	58.0	141.0	246.3	453.3	565	60	470	530	580	730	810
M (28-63)	5.8	69.5	144.5	269.0	428.0	..	80	570	730	840	900	..
P (26-75)	0	88.4	181.9	275.3	353.8	405.5	20	120	300	400	510	560
E (39-77)	14.8	101.8	229.6	307.8	465.0	550.5	190	790	970	1,060	1,240	1,390
S (33-57)	14.5	123.2	232.4	308.4	414.0	495.2	60	430	690	790	900	1,040
N (21-77)	2.0	50.1	106.8	166.3	250.3	305.7	80	500	570	650	770	910
I (33-61)	42.5	196.9	277.2	363.1	442.3	589.1	40	170	270	340	410	570
Mean	12.1	98.3	187.6	276.6	401.0	485.2	76	436	580	666	780	880

[a] Values shown are for free drug. Only negligible amounts of acetylated drug were excreted to the longest time shown.
[b] Bracketed quantities are subject's age in years followed by his weight in kilograms.

Fig. 3. Values for the excretion rate term of Eq. 3 were obtained from a similar plot only with an expanded scale for excretion between zero and three and one-half hours. These values were mean rates over twelve-minute intervals having the times listed in Table IV as their midpoints. The rates obtained by this graphical determination are shown in the second column of Table IV. The second derivative terms for Eq. 3 were obtained by plotting these rates *vs.* time and were taken over the same time interval. The values obtained are listed in the third column of Table IV. The value of the constant K in Eq. 3 was obtained from the mean half-lives for elimination of sulfaethylthiadiazole by the subjects as determined by plotting their graphically determined individual excretion rates, at four and one-half, five and one-half, six and one-half, and seven and one-half hours after drug ingestion, as a function of time on semilogarithmic paper.[5] The half-lives so obtained are listed in Table V. The value of the constant K obtained by use of the mean half-life was 0.091 hours^{-1}. The only other quantity needed to calculate absorption rate was the value of f which was taken at 0.92 since this value appears in the literature as the fraction of this drug in circulation that is excreted as unchanged material (41) The calculated absorption rate in mg./hour at various times is shown in column 4 of Table IV.

TABLE IV.—ABSORPTION RATE OF SULFAETHYLTHIADIAZOLE FROM URINARY EXCRETION DATA AND BLOOD LEVEL DATA[a]

Time, hr.	Excretion rate, mg./hr.	Derivative of Excretion Rate, mg./hr.[2]	Absorption Rate from Excretion Data,[b] mg./hr.	Absorption Rate from Blood Data,[b] mg./hr.
0.25	20	107	1,200	500
0.50	50	105	1,210	1,230
0.75	72	83	1,000	1,170
1.00	87	55	700	830
1.25	100	35	490	250
1.50	112	0	120	90

[a] Derived data needed to calculate absorption rate from urinary excretion data are also shown. The calculation in the fourth column was made by Eq. 3.
[b] Nearest 10 mg./hr.

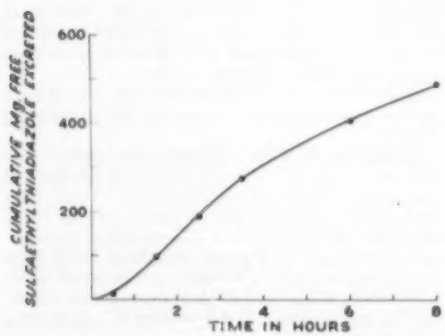

Fig. 3.—Mean cumulative excretion curve for sulfaethylthiadiazole after ingestion of 1.0-Gm. doses by seven subjects. Drug taken in suspension.

TABLE V.—HALF-LIVES FOR ELIMINATION OF SULFAETHYLTHIADIAZOLE BY TEST SUBJECTS[a]

Subject	Half-Life, Hours
O	9.7
P	3.1
E	9.1
S	7.8
N	5.4
I	10.8
Mean	7.6

[a] See text for method of determination.

A method of calculating absorption rate based on blood level measurement at short-time intervals following drug ingestion (17) was applied to data in the literature obtained following the same dose of sulfaethylthiadiazole (41). The blood level data on eight normal human subjects were averaged and the absorption rate calculated at the same times with rates determined over the same time intervals as in the calculation based on Eq. 3. The values so calculated are shown in column 5 of Table IV.[6] The agreement in calculated rates was surprisingly good. A different particle size distribution may have existed in the two sets of dosage forms, but the sulfaethylthiadiazole was from the same manufacturer. The conditions of administration differed to the extent that, while subjects in both groups took the drug on fasting stomachs, the drug was ingested in suspensions for urinary excretion measurements and in capsules for blood level measurements. The lower value in absorption rate calculated at fifteen minutes (Table IV) from capsule dosage may reason-

[5] Excretion rates at earlier times were not used in this plot to avoid introduction of error due to continued absorption of small amounts of drug and/or nonequilibrium conditions between blood and other distribution fluids.
[6] The data used were from Table I of (41) for 1.0-Gm. doses. Other constants used in this calculation which were in accord with information in the cited paper were: Vb = 15,000 ml. (based on mean body weight of subjects) and kb = 0.092 hours^{-1}. The quantities, Vb and kb, are in the terminology of (41).

ably be attributed to a five to ten-minute delay in the capsules' contents reaching the same state of dispersion *in vivo* obtained when the drug was taken in suspension.

The area under an absorption rate *vs.* time curve plotted from data obtained by Eq. 3 gave an apparent amount of drug absorbed equal to 1.17 Gm.

SUMMARY

The theoretical bases for application of urinary excretion data to evaluation of drug absorption were reviewed and the concepts applied to study (*a*) the absorption of 0.65-Gm. doses of aspirin in solution and in rapidly disintegrating tablets, (*b*) the absorption of 100,000 units of benzyl penicillin from the potassium and procaine salts given in thin pellets of limited surface area wherein *in vivo* solution rate, rate-limited absorption, and (*c*) the absorption rate of sulfaethylthiadiazole following 1.0-Gm. doses. All studies were conducted in normal humans after oral ingestion of drugs. The absorption of aspirin appeared to be rate limited by the time necessary for this drug to dissolve after ingestion and this finding was in accord with theoretical calculations previously made which indicated that this process should be rate limiting in absorption. The absorption of benzyl penicillin was found to be rate limited by the intrinsic solution rate properties of the potassium and procaine salts used. The *in vivo* solution rates of these two salts were considered on a physical chemical basis and an expression derived to relate rate to diffusion layer pH. From excretion studies with sulfaethylthiadiazole it was calculated that this drug was absorbed fifteen to thirty minutes after ingestion, at a rate of about 1,200 mg./hour. This value was in excellent agreement with absorption rate calculated from blood level data appearing in the literature.

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The Effect of Gibberellic Acid on the Second-Year Growth and Alkaloid Formation in *Atropa belladonna* Linné*

By LEO A. SCIUCHETTI

Atropa belladonna **treated in 1957 with gibberellic acid in concentrations of 100 and 1,000 p. p. m. was allowed to overwinter. The second-year crop of the treated plants generally did not demonstrate the "gibberellin effects" noted in the first-year growth following treatment. In some instances reverse effects were noted. The plants previously treated with 100 p. p. m. of gibberellic acid generally closely resembled the controls in their growth and alkaloid patterns. However, belladonna treated the previous year with 1,000 p. p. m. of gibberellic acid was profoundly affected. This treatment induced about a 50 per cent decrease in total dry weight and approximately a 15 per cent decrease in height. The concentration of alkaloids in the leaves and roots was not markedly altered, whereas that in the stems was increased about 44 per cent. The total alkaloid content of this group was about 56 per cent that of the controls.**

IN A PREVIOUS PUBLICATION (1) it was demonstrated that the growth and alkaloid content of *Atropa belladonna* were adversely affected by treatment with gibberellic acid (G. A.). This study was a continuation of that research in that the remaining plants (treated and untreated) were allowed to overwinter. The objective of this research was to observe whether the characteristic "gibberellin effects" would again be manifested in the second-year growth.

EXPERIMENTAL

Procedure.—Fifty-four belladonna plants remaining from the previous study (1) were allowed to overwinter in the drug garden. The plants were divided into three groups of 18 plants each. The first group constituted the control or untreated plants; the second were those subjected in 1957 to a three-spray treatment with 100 p. p. m. of G. A.; and the third were those similarly treated with 1,000 p. p. m. of G. A. The first-year growth continued until mid-December, 1957, when a killing frost occurred. Several weeks of freezing temperatures were followed by about a month of warm moist weather. Due to the mild winter new shoots appeared during the second week in February, 1958. Height measurements were started about two months later. On June 3, 1958, ten plants were picked at random from each group and the shoots were harvested. At this time the plants were at the flowering stage. This constituted the first crop.

Following the first harvest the plants were fertilized by the application of a complete organic fertilizer[1] to the plot containing the plants. Height measurements for the second crop were commenced on June 23. The plants were harvested on September 9. At this time the plants were in the flowering and fruiting stage, i. e., many of the plants had green berries.

The first crop, consisting of the shoots, was harvested and divided into two portions—leaves (with flowering tops) and stems. Fresh weights were immediately taken of each portion and the fresh material was promptly transferred to a hot-air circulating drier. The plant parts were dried at a temperature of 48.5° for forty hours. Upon removal from the drier the plant parts were placed in a desiccator, allowed to attain room temperature, and then weighed. The portions were stored in air-tight metal containers until subsequent pulverization into a No. 40 powder in a Wiley mill. The powdered material was then stored in air-tight colored-glass containers until subsequent analyses for alkaloids were performed. For the second crop, an additional (third) portion which consisted of the roots was harvested. Otherwise, the harvesting procedure was identical with that for the first crop.

Growth Observations.—New shoots mainly in the control group were first observed during the second week of February, 1958. All of the control plants survived the winter. One plant from the group treated with 100 p. p. m. of G. A. and two from the group treated with 1,000 p. p. m. of G. A. did not overwinter. In general, the untreated plants and those previously treated with 100 p. p. m. of G. A. closely resembled each other in growth characteristics during the growing season. However, those previously treated with 1,000 p. p. m. of G. A. did not commence to form shoots from the first-year root stocks until about two weeks after the controls. Growth in this group was slower and the onset of flowering was later. Also, the type of growth was somewhat different in that the plants were spindly and vine-like and the leaves were narrower and thinner.

On April 16, 1958, height measurements were taken of all the plants. These measurements were taken weekly thereafter at approximately the same time of the day. For each crop, the control plants and those treated with 100 p. p. m. of G. A. grew to approximately the same height (Fig. 1). However, the first crop of the group treated with 1,000 p. p. m. of G. A. attained a height of about 61% of the controls at the start of measurements and about 83% of the controls when harvested. The second crop of this group attained about the same height as the controls at the start of measurements on June 23, but

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[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis—50% of total nitrogen, 3% available phosphate, 2% available potash.)

were about 85% as tall as the controls when harvested (Fig. 1).

Correlation With Fresh and Dry Weight Data.—The treated plants displayed decreased dry weights in their second-year growth (Table I). The plants previously treated with 100 p. p. m. of G. A. displayed slight decreases of about 8 to 10% in the dry weights of their organs. This consistency was noted for both crops. Significant decreases, however, were observed in the plants previously treated with 1,000 p. p. m. of G. A. Their total dry weight was about one-half that of the controls. The decreases were considerably greater for the plants of the second crop. Also, the roots displayed the largest decrease among the organs of this group (Table I).

Analysis for Alkaloids.—The dried plant parts, using pooled samples, were assayed for total alkaloids (expressed as hyoscyamine) according to the Witt-Youngken method (2), substituting chloroform for benzene as the immiscible solvent. The concentration of alkaloids in the various plant organs of the group treated with 100 p. p. m. of G. A. differed slightly from that of the controls (Table II). This was also true for the leaves and tops and the roots of those treated with 1,000 p. p. m. of G. A. However, significant increases were found in the stems of this group. Compared with controls, the increases were about 28% and 54% for the first and second crops, respectively. The increase for the total crop of stems was about 44%.

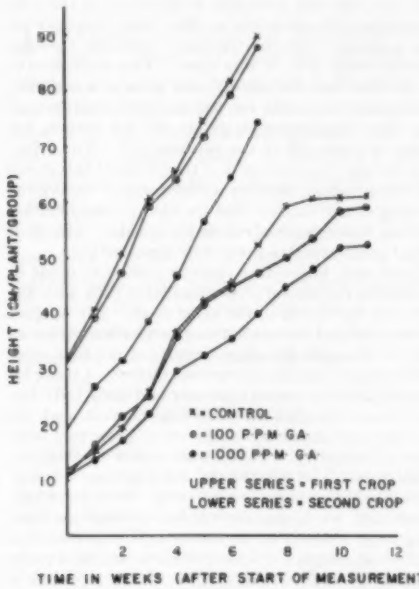

Fig. 1.—Increase in height of *Atropa belladonna*.

It was further desired to make comparisons based on the total alkaloid content per plant and per plant organ. The total alkaloid content was obtained by multiplying the dry weight of the plant organ by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in mg. (Table II). All of the plants treated with 100 p. p. m. of G. A. contained less total alkaloids than the controls. The decreased alkaloid content of this group was not considered significant. Significant decreases, however, were found in the plants treated with the higher concentration of gibberellic acid. In general, the total alkaloid content of this group was about one-half that of the controls. The roots displayed the greatest decrease among the plant organs, *viz.*, 57% less than the controls.

DISCUSSION

Characteristic "gibberellin-effects" were demonstrated in belladonna subjected in 1957 to three-spray treatments of G. A. in concentrations of 100 and 1,000 p. p. m. (1). Among the specific effects reported for the treated plants were: increased stem elongation which resulted in a two- to three-fold increase in height; a spindly and vine-like type of growth; leaves which were slightly chlorotic, narrower, and thinner; more rapid onset of flowering; significant decreases in leaves and tops and root dry weights accompanied by significant increases in stem weights; no significant changes in total dry weights. Also, significant decreases in the concentration of alkaloids were noted in the plant organs. The total alkaloid content of the treated plants amounted to about one-half that of the controls.

Belladonna receiving a treatment with 100 p. p. m. of G. A. in 1957 generally did not demonstrate the aforementioned "gibberellin-effects" in their second-year growth in 1958. In several instances the re-

TABLE I.—WEIGHTS OF SECOND-YEAR GROWTH OF *Atropa belladonna* (AV./PLANT/GROUP)

Treatment	Total Weight			Leaves and Tops			Stems			Roots		
	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %
Control												
First harvest	247.2	33.3	..	123.2	16.8	..	124.0	16.5	..	..	..	..
Second harvest	581.3	104.5	..	257.8	39.4	..	162.5	23.9	..	160.9	41.2	..
Total	828.5	137.8	..	381.0	56.2	..	286.5	40.4	..	160.9	41.2	..
100 p. p. m. G. A.												
First harvest	228.2	30.7	92.2	112.4	15.5	92.3	115.8	15.2	92.1	..	..	..
Second harvest	540.8	95.4	91.4	254.1	36.1	90.6	136.9	21.5	90.0	149.8	37.8	91.7
Total	769.0	126.1	91.5	366.5	51.6	91.8	252.7	36.7	90.8	149.8	37.8	91.7
1,000 p. p. m. G. A.												
First harvest	166.5	20.7	62.2	85.4	11.1	66.1	81.0	9.6	58.2	..	..	..
Second harvest	318.4	48.5	46.4	157.0	20.3	50.5	82.4	10.7	44.8	79.1	17.5	42.5
Total	484.9	69.2	50.2	242.4	31.4	55.9	163.4	20.3	50.2	79.1	17.5	42.5

TABLE II.—ANALYSIS OF ALKALOIDS[a] IN BELLADONNA

Treatment	Total Alkaloids, mg./Gm.			Total Alkaloid Content,[b] mg.			
	Leaves and Tops	Stems	Roots	Per Plant	Leaves and Tops	Stems	Roots
Control							
First crop	3.44	3.80	...	120.5	57.8	62.7	...
Second crop	4.20	1.98	3.47	355.8	165.5	47.3	143.0
Total crop	3.97[c]	2.72[c]	3.47	476.3	223.2	110.0	143.0
100 p. p. m. G. A.							
First crop	3.50	3.59	...	109.0	54.3	54.7	...
Second crop	4.22	2.13	3.33	324.0	152.3	45.8	125.9
Total crop	4.00[c]	2.74[c]	3.33	433.0	206.6	100.5	125.9
1,000 p. p. m. G. A.							
First crop	3.53	4.87	...	86.0	39.2	46.8	...
Second crop	4.19	3.05	3.54	179.7	85.1	32.6	62.0
Total crop	3.96[c]	3.91[c]	3.54	265.7	124.3	79.4	62.0

[a] Total alkaloids calculated as hyoscyamine. [b] Alkaloid content for plant parts calculated from dry wt. and alkaloid analyses data; total plant alkaloid = leaves and tops + stems + roots. [c] Obtained by calculation.

verse effect was noted. Compared with controls, the treated plants were shorter, had decreased rather than increased stem weights, the onset of flowering was slower, and the concentration of alkaloids in the plant parts and the total alkaloid content did not change significantly. In general, this group of plants closely resembled the controls.

A few significant differences were noted, however, in the group previously treated with the higher concentration (1,000 p. p. m.) of G. A. In addition to the aforementioned reversals, significant decreases in total dry weight and in the weights of the plant organs were noted. The total dry weight of the second-year crop of this group was about 50% of that of the controls. At each harvest the treated plants attained a height of about 85% of that of the controls. The concentration of alkaloids in the leaves and roots was about the same as the controls. However, the quantity of total alkaloids in the stems of the second-year crop was about 44% greater than that of the controls. This effect was the reverse of that observed in the first-year crop, since in that case, a 57% decrease was observed. It should be pointed out that following treatment in 1957 the increased stem elongation resulted in significant increases in plant height and stem dry weights. The second-year growth demonstrated the reverse trend—shorter plants and significantly decreased stem weights. It is possible that in this case there was less stem area to dilute a fixed amount of alkaloid biosynthesized by the roots; or, since the active growth shown by the stem tissues in 1957 did not occur in the second-year crop, there was not a diversion of nitrogenous material which might be used for stem elongation and growth, but, rather was used for alkaloid biosynthesis. It was also observed during both harvests of the 1958 crop that the stems of this group were less woody and the plants had matured more slowly than those of the other groups. The above factors might possibly explain the increased concentration of alkaloids in the stems.

The most striking change in the second-year crop was that the plants previously treated with the higher concentration of G. A. had a total alkaloid content about one-half that of the controls. This was due primarily to approximately a 50% decrease in dry weight of this group. It is true that the first year crop of this group demonstrated a similar trend but the 50% decrease in total alkaloid content, in this case, was due primarily to decreases in the concentration of alkaloids in the plant organs. It also appeared that the treatment with the stronger concentration of G. A. was toxic. This is attested to by the fact that the second-year growth was significantly less than that for the untreated plants and that two of the eighteen plants did not survive the winter whereas all of the controls did. These factors and the observation that the plants of this group did not appear as healthy as the controls during the growing season indicate that the higher concentration of G. A. had a toxic effect on the plants. This confirms the observation previously reported (1).

Some very interesting observations were made in comparing the second crop obtained in 1958 with the first crop harvested in the same year. The weights of the shoots of the second crop were about twice as large as the corresponding weights of the first crop. This increase was due to several factors: a more favorable growing season (summer and early fall), fertilization of the plot after the first harvest, and the fact that the plants were allowed to grow to a later stage of maturity. The concentration of alkaloids in the leaves and tops of the three groups was significantly higher in the second crop. This was unexpected since the first group was harvested at the flowering stage whereas the second crop was harvested at the fruiting stage, i. e., many of the plants had green berries. Several workers (3–6), have reported a higher content of alkaloids at the flowering stage than at either an earlier or later stage of development. Evans and Partridge (6) have reported that the concentration of alkaloids is the highest during flowering but declines during fruiting for both the first- and second-year growth of belladonna. Our results are not in agreement with these reports. However, it is possible that collecting two harvests from the second-year growth may have been a factor. The advanced maturity of the stems as indicated by their woody character explains the significant decrease in the concentration of alkaloids in the stems of each group.

When the total 1958 crop was compared with the single 1957 crop the following differences were found: a 28-fold increase in the total dry weights of the control plants and those treated with 100 p. p. m. of G. A. compared with a 14-fold increase in the plants treated with the higher concentration of G. A.; the concentration of alkaloids in the controls increased

about 18% in the leaves and tops and decreased 36% in the stems and 20% in the roots; the corresponding figures for the plants previously treated with 100 p. p. m. of G. A. were approximately a 108% increase, a 22% increase, and a 7% decrease; those for the plants treated with 1,000 p. p. m. of G. A. were increases of about 113%, 112%, and 7%, respectively.

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The Infection of Rye Callus With *Claviceps purpurea**

By DAVID P. CAREW and ARTHUR E. SCHWARTING†

The methods and results of inoculating callus cultures of rye (*Secale cereale*) with ergot (*Claviceps purpurea*) are described. The fungus grows intercellularly and invades some cells, but does not produce alkaloids.

THE ARTIFICIAL CULTURE of *Claviceps purpurea* (Fries) Tulasne for successful alkaloid production is a problem which has been widely investigated. The majority of these investigations have been concerned with the growth of *C. purpurea* on synthetic media to obtain an understanding of the factors concerned with alkaloid formation.

Recently the present authors reported the successful production and maintenance of a callus culture of rye, *Secale cereale L.*, on artificial media (1). Since rye is a natural host for ergot, this tissue was used in this study to investigate certain aspects of the host-parasite relationship.

EXPERIMENTAL

The host tissue consisted of a mass of callus cells which had originally been produced from fifteen to twenty-day-old embryos of rye (*Secale cereale*) growing on Heller's modified media (1) solidified with 1.0% agar. The tissue cultures had been maintained and transplanted to fresh media at approximately twenty-five day intervals for more than a year.

The rye callus was transplanted to agar plugs in 25 mm. x 150 mm. Pyrex culture tubes. A sterile, Pyrex glass cylinder, approximately 1.25 cm. long and 15 mm. in diameter, was inserted into each tube, in a position surrounding the callus and was made to penetrate the agar surface. Growth continued for approximately fifteen days, until the lateral callus growth covered the agar surface within the cylinder. This design prevented the inoculum, when introduced, from coming into direct contact with the agar.

The inoculum was prepared as follows: mycelial growth of *Claviceps purpurea* strain RCNA2, obtained from mature rye sclerotia and maintained on nutrient agar, was used to inoculate a liquid medium consisting of casein hydrolysate, 10.0; mannitol, 20.0; KH_2PO_4, 2.5; and $MgSO_4 \cdot 7H_2O$, 1.25 Gm./L. distilled water. The inoculated liquid media was grown as a submerged culture for eight days to provide inoculum for the callus growth.

The inoculum, 0.4 ml., was delivered to the surface of the callus growth. The tubes were plugged with cotton, capped with aluminum foil, and incubated in the dark, in a vertical position at 25°.

Infected callus was removed from the tubes at various intervals during forty days. In each case, the tissue was fixed in formalin-acetic acid-alcohol, dehydrated, and embedded in paraffin. The embedded tissue was sectioned with a rotary microtome and the sections stained in safranin-fast green. Infected tissue, of equivalent age, was dried, defatted, extracted in the usual manner, and qualitatively tested with Ehrlich's reagent. After forty days a quantity of tissue, obtained by pooling the infected tissue of a number of tubes, was dried at 32° in a circulating-air oven. The product was extracted and assayed (2) for total alkaloids.

EXPERIMENTAL RESULTS

Microscopic examination of the inoculated callus revealed the early intracellular growth of ergot mycelium. The penetration of occasional cells occurred within twenty-four to forty-eight hours. The arrow in Fig. 1 is directed to a penetrated cell wall. Extensive intercellular growth was always observed and can be seen among the rye callus cells in the figure. The majority of mycelial growth was superficial to the callus structure.

Extracts of the infected callus, when tested quali-

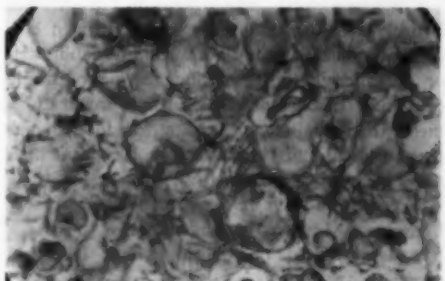

Fig. 1.—Rye callus infected with *C. purpurea*. Arrow indicates a single cell penetrated by the fungus.

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tatively with Ehrlich's reagent, consistently gave negative tests for alkaloids. The larger quantity of tissue, 2.813 Gm., dry weight, contained no alkaloids upon quantitative assay.

SUMMARY AND CONCLUSIONS

A callus culture of rye readily supports the growth of ergot and there is definite cellular penetration by the fungus. While callus is a satisfactory host for the fungus, there is no evidence of alkaloid formation during prolonged host-parasite association.

The early penetration and the intracellular invasion of rye callus observed here is analogous to the natural infection phenomenon (3, 4). The results, however, are contrary, in part, to those (5) reported on the growth of ergot on intact and punctured wheat root and rye embryo cultures.

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The Reduction of Hydrolysis Time and Tryptophan Destruction in the Preparation of Casein Hydrolysates*

By VINCENT S. VENTURELLA†, ROBERT W. SAGER, and JOSEPH A. BIANCULLI

A method was developed for hydrolyzing casein using a procedure which reduces the time requirements of enzymatic protein hydrolysis and minimizes the tryptophan destruction encountered in acid protein hydrolysis. Hydrolysates prepared by this method in twenty-four hours or less are comparable to enzymatic hydrolysates requiring one hundred hours. The method involves the treatment of partial enzymatic hydrolysates with diluted acids.

THE IMPORTANCE of protein hydrolysates in the treatment of certain deficiency symptoms and related conditions is well known. Such conditions as impaired digestion or absorption, poor assimilation because of liver damage, febral states, leukemia, hemorrhage, severe operations, burns, fractures, or shock usually result in an increased loss of amino acids and proteins (1, 2).

The parenteral administration of protein hydrolysates to maintain positive nitrogen balance is indicated in patients who are debilitated or are allergic to the administration of whole protein. It is essential that these preparations meet the usual requirements for intravenous preparations, and they should not contain high molecular weight peptides (2).

If a protein such as casein could be partially hydrolyzed by enzymatic methods to a low molecular weight polypeptide stage, it would seem possible that treatment with diluted acid should hydrolyze these polypeptide chains to even smaller peptides in a shorter period of time than would be needed by using enzymes alone. The amount of tryptophan destruction should also be less than that ordinarily observed with the use of strong acid alone.

* Received May 18, 1959, from the School of Pharmacy, University of Pittsburgh, Pittsburgh 13, Pa.
Abstracted in part from a thesis submitted to the Graduate School of the University of Pittsburgh by Vincent S. Venturella in partial fulfillment of the requirements for the degree of Master of Science.
† George A. Kelly, Sr., Fellow, 1954–1955.

EXPERIMENTAL

The moisture content of acid-washed casein,[1] determined in duplicate, was found to be 7.78%. All analytical results are calculated on the basis of dry casein.

Analytical Procedures.—*Total Nitrogen.*—The total nitrogen present in the casein sample was determined using a modification of the semimicro Kjeldahl method described in the United States Pharmacopeia (3). The method was modified by using 0.5 Gm. each of copper sulfate and potassium sulfate, together with a Hengar selenized granule. The average total nitrogen content of the casein sample was 15.41%.

Amino Nitrogen.—The amino nitrogen content of whole casein and of samples of hydrolysates was determined using a modification of the Sorenson formol titration described by Sahyun (4). A line operated pH meter, Beckman Model H, No. 487 was used to determine the necessary end points. Sodium hydroxide solution, 0.2 *N*, was used as the base. The amount of amino nitrogen was calculated using the following equation: $(A - B) \times 2.8$ mg. = mg. of amino nitrogen, where A = volume of sodium hydroxide solution for sample, and B = volume of sodium hydroxide solution used for the blank.

Using the average per cent total nitrogen present in the casein samples, the theoretical amount of amino nitrogen present at complete hydrolysis was

[1] Fisher Scientific Co., No. C-200.

calculated. This calculation was made from data obtained in an experiment in which 20 Gm. of casein was digested for twenty-six hours in refluxing 6 *N* sulfuric acid. A sample of casein so treated was found to contain 12.59% amino nitrogen. A comparison of amino nitrogen and total nitrogen showed that the percentage ratio of amino nitrogen to total nitrogen was 81.7%. This is the percentage of total nitrogen present as amino nitrogen.

The experimental conditions (5, 6), the results of this amino nitrogen analysis, and the nitrogen ratio obtained (7) were consistent with literature reports. Further evidence that the casein had been completely hydrolyzed was obtained by digesting the casein under identical conditions for two more hours. The amino nitrogen content of the hydrolysate did not change.

Colorimetric Determination of Tryptophan.—The amount of tryptophan present in the samples, presumably in the free or simple peptide stage, was determined colorimetrically by an adaption of Winkler's modification of the Hopkins-Cole method (8). The glyoxylic acid employed in the color reagent was prepared by reduction of oxalic acid with magnesium (8). In each case throughout the study, a 0.3-ml. sample of the hydrolysis mixture was mixed with 1.5 ml. of the color reagent. The volume of the mixture in each sample tube was adjusted to 9.5 ml. with deionized water prior to the addition of 15 ml. of sulfuric acid. The treated samples were placed in 2.5-cm. diameter cuvettes and the transmittance was determined with a Fisher electrophotometer, using a 525 mμ filter. Deionized water was used as the blank.

To ascertain the validity of the Lambert-Beer law in this determination, known concentrations of L-tryptophan solutions were determined colorimetrically. The results showed good conformity to the law by the production of a straight line plot when using concentration in μg. as ordinate and log I/I_0 as abscissa. Thus it was possible to calculate concentration of unknowns by means of the following equation, which is a form of the Lambert-Beer law (9): $\log I/I_0 = -a_s bc$, where a_s is the absorbance index, b is the diameter of the cuvette, and c is the concentration. The per cent transmission values of various concentrations of a series of samples of L-tryptophan appear in Table I. For the series of known values plotted, a value of 5.57×10^{-4} μg.$^{-1}$ cm.$^{-1}$ was calculated for the absorbance index.

TABLE I.—TRANSMISSION OF LIGHT OF THE COLORED REACTION PRODUCT OF CRYSTALLINE L-TRYPTOPHAN AND GLYOXYLIC ACID AT 525 mμ

L-Tryptophan, μg.	Transmission, %	Log I/I_0
28.8	88.6	−0.054
57.5	85.0	−0.070
115.0	72.9	−0.138
201.3	53.5	−0.272
287.5	40.7	−0.390
373.8	31.5	−0.503

Electrophoretic Tryptophan Determination.—The concentration of tryptophan was also determined for a number of the hydrolysates by the use of paper electrophoresis as a check on the colorimetric method. This method was possible because an amino acid in solution at a pH other than at its isoelectric point will migrate under the influence of an electric force. Paper electrophoresis makes use of this property (10, 11).

Two series of experiments were performed using a Reco Model E-800-2 migration chamber and power supply. This instrument operates on the horizontal "open-strip" method (11). The position of the amino acid spot was determined by developing with 0.1% ninhydrin in 95% ethanol in the usual manner (12). The color density curves of the samples of crystalline L-tryptophan and tryptophan isolated from the hydrolysate samples, according to the method of Cox and King (13), were determined using a Photovolt densitometer, model 425. To compare the amount of crystalline L-tryptophan against the amount of tryptophan isolated by this method, it was necessary to compare the area under the standard curve with the area under the curve obtained from the unknowns. This was accomplished by applying the trapezoidal rule (14) to a plot of the absorbance as abscissa against the migration distance as ordinate.

The hydrolysate samples were placed on Whatman No. 3, 1½-inch strips as the support. Buffers of 5 *N* acetic acid (15), pH 1.8, and 0.1 *M* phosphate, pH 3.5, were used in two successive experiments. When 5 *N* acetic acid was used, a current of 0.33 ma. at a potential of 500 v. was applied for two hours. This produced a migration of 2.9 cm. In the experiment employing the phosphate buffer, a potential of 450 v. was applied for the same period of time using a current of 3.5 ma. The migration in this case was 3.9 cm. The migration in both cases was toward the cathode as shown in Fig. 1.

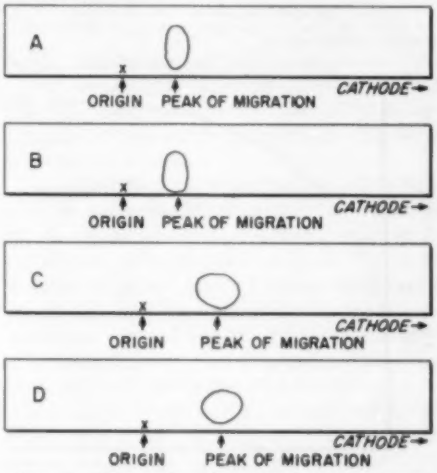

Fig. 1.—Electrophoretic migration of L-tryptophan. Strips A and B using 5 *N* acetic acid, pH 1.8. Strips C and D using 0.1 *M* dibasic sodium phosphate-phosphoric acid, pH 3.5. Strips A and C represent crystalline material. Strips B and D represent isolated material.

The results of a few comparisons appear in Table IV. The results obtained electrophoretically when compared to the colorimetric results show good correlation of the two methods.

Hydrolytic Methods.—*Enzymic Hydrolysis.*—

The enzymatic digestion of casein was studied in order to determine the relative rate of release of amino nitrogen. This was done because it was the partial enzymatic hydrolysates showing the most uniform release of amino nitrogen that would be chosen for further study.

One hundred grams of casein, suspended in 690 ml. of deionized water, was mixed with 6 Gm. of U. S. P. pancreatin. The pH was adjusted to 8.5 with 4 *N* sodium hydroxide solution. The suspension was stirred for one hour, and then adjusted to pH 7.9–8.0. It was then diluted to 1,000 ml., toluene was added to just cover the top of the suspension, and then the mixture was incubated at 37°.

Ten-milliliter portions of the hydrolysates were periodically removed, and after each removal the pH of the suspension was adjusted to 8.0. The amino nitrogen content of each portion was determined. The rate of enzymatic hydrolysis was thus studied by plotting the mg. of amino nitrogen released against time of hydrolysis. The results of the analyses are tabulated in Table II and shown graphically in Fig. 2.

TABLE II.—ENZYMATIC HYDROLYSIS

Hours of Digestion	Amino Nitrogen Released[a]	Nitrogen Ratio[b]
0.0	14.8	0.117
4.5	28.4	0.225
20.0	35.1	0.280
26.3	38.1	0.304
29.5	38.6	0.307
51.3	44.0	0.350
99.3	44.7	0.353

[a] Mg. per Gm. of dry casein.
[b] Based upon the ratio of amino nitrogen/total nitrogen in complete acid hydrolysis.

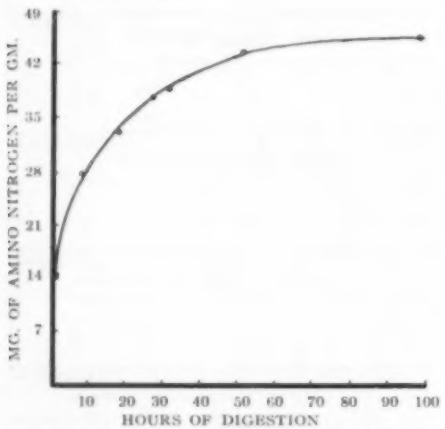

Fig. 2.—Rate of amino nitrogen release in the pancreatic digestion of casein.

The hydrolysates chosen as the ones presenting the most uniform release of amino nitrogen were thirteen to twenty-four hour hydrolysates. These hydrolysates were the ones chosen for the modified hydrolytic procedure.

Acidic Hydrolysis.—A casein sample was completely digested with 6 *N* sulfuric acid as previously described, to determine the amino nitrogen present on complete hydrolysis and to determine, colorimetrically, the amount of tryptophan present. In this experiment, a sample of the hydrolysate itself was used as the blank since the hydrolysate was slightly discolored. This experiment yielded 12.59% amino nitrogen based upon the dry weight of casein. The sample contained 0.06% tryptophan.

Modified Enzymatic and Acidic Hydrolysis.—A modified hydrolytic procedure was then used with a series of enzymatic hydrolysates, the time of hydrolysis falling in the range of uniform release of amino nitrogen. At the end of thirteen to twenty-four hours of enzymatic hydrolysis, the mixture was heated on a water bath at 80° for ten minutes to deactivate the enzyme. After the suspension cooled, 10 Gm. of charcoal was added to partially decolorize the hydrolysate and it was filtered at reduced pressure through a Büchner funnel.

A sample of the filtrate so obtained was analyzed for amino nitrogen and tryptophan. Five hundred milliliters of the filtrate was then mixed with 500 ml. of the appropriate diluted sulfuric acid solution and refluxed at 103° for the prescribed interval of time. At the end of this time, the solution was cooled and the amino nitrogen content and tryptophan content again determined.

A summary of the amino nitrogen determinations and tryptophan analyses, before and after acid treatment, with nitrogen ratio, appears in Table III.

TABLE III.—RESULTS OF THE MODIFIED HYDROLYTIC PROCEDURES

Hours of Hydrolysis Enzyme	Hours of Hydrolysis Acid	Molarity of Acid	Amino Nitrogen[a]	Tryptophan, %	Nitrogen Ratio
13	..	..	15.0	0.54	0.119
13	2	0.2	17.7	0.66	0.141
13	3	0.2	24.4	0.52	0.194
13	2	0.3	18.2	0.55	0.145
16	..	..	20.5	0.59	0.163
16	2	0.2	25.8	0.94	0.205
16	3	0.2	26.0	0.92	0.207
16	2	0.3	26.0	0.88	0.207
17	..	..	34.2	0.98	0.272
17	2	0.2	46.0	1.21	0.366
17	2	0.1	43.3	1.05	0.344
17	2	0.3	50.9	0.92	0.405
20	..	..	31.5	0.96	0.250
20	2	0.2	40.8	1.26	0.324
22	..	..	34.6	0.95	0.275
22	2	0.2	44.6	1.36	0.352
24	..	..	34.9	0.79	0.278
24	2	0.2	46.7	0.79	0.372

[a] Mg. per Gm. of dry casein.

TABLE IV.—COMPARISON OF % TRYPTOPHAN OBTAINED COLORIMETRICALLY AND ELECTROPHORETICALLY

Hours of Hydrolysis Enzyme	Hours of Hydrolysis Acid	Acid Molarity	Tryptophan, % Colorimetric	Tryptophan, % Electrophoretic
16	2	0.2	0.94	0.87
17	2	0.2	1.21	1.09
20	2	0.2	1.26	1.15
24	2	0.2	0.79	0.74

Av. % Difference—0.08
Av. % Deviation—7.60

SUMMARY AND CONCLUSIONS

The results of the method described to decrease both the time of hydrolysis and the destruction of tryptophan in preparing casein hydrolysates indicates the potential value of the method. The data tabulated in Table III suggest that hydrolysis with pancreatin for seventeen to twenty-two hours followed by refluxing with 0.2 *M* sulfuric acid for two hours, results in a product containing about 45 mg. of amino nitrogen per Gm. of casein and about 1.3 per cent tryptophan. These products represent a nitrogen ratio of about 0.36.

The desirability of this method arises from the saving in time because the degree of hydrolysis obtained, although no greater than commercial enzymatic products, was attained in seventeen to twenty-two hours, whereas it was noted that one hundred hours digestion with pancreatin alone is necessary to attain the same result. Commercial acid hydrolysates usually exhibit a greater degree of hydrolysis with a concurrently greater destruction of tryptophan.

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Substituted Salicylanilides With Antimicrobial Activity*

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Eighteen new halonitrosalicylanilides have been prepared. Of these the salicylanilides of 3-nitro- and 5-nitrosalicylic acid, halogenated by one, two, or three halogens of various kinds in the aniline nuclei, have been shown to possess a high degree of bacteriostatic and fungistatic activity.

SALICYLANILIDE, a widely-used commercial fungistat, was first recognized by Fargher and co-workers (1) to be an excellent textile preservative. Derivatives of salicylanilide have been finding increasing mention in the literature. Thus, apparently in trying to widen the range of antimicrobial activity, interesting compounds such as those wherein the salicylanilide moiety and sulfonamide moieties have been united (2–5) or an anilide made from *p*-aminosalicylic acid (6–12) have been examined. However, the former compounds were not reported to have exceptionally better antimicrobial properties than salicylanilide or the sulfonamides alone, and the latter were not reported as being superior to *p*-aminosalicylic acid as tuberculostats. Recently, polyhalogenated salicylanilides have received considerable attention (13–15), it being reported that particularly 5-bromo-4′-chlorosalicylanilide has a high potency and wide range of antimicrobial action.

Salicylanilide and substituted salicylanilides have been previously prepared in low yields by the direct condensation of salicylic acid, or the substituted acid with aniline, or a substitution derivative of aniline (16). Various condensation agents such as aluminum anilide (17), phosphorus trichloride (11, 14–16, 18, 19), and tetralkyl pyrophosphites or dihalophosphites (20) have been used to increase the efficiency of the reaction greatly. These compounds have also been prepared in good yields by the reaction of the phenyl or methyl esters of the particular salicylic acid with the appropriate aniline (14, 21). Salicoyl chloride and substituted salicoyl chlorides have been reacted with various anilines to give the salicylanilides (14, 22). The acid chlorides have been prepared by classical means without the necessity of blocking the *ortho* hydroxyl group (23, 24). Of a number of other methods which are less orthodox and generally less efficient, two are particularly interesting.

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Thus, *ortho*-carbamyl derivatives of diphenyl ethers have been rearranged to salicylanilides (25). Further, the sodium derivative of the phenyl ester of phenylcarbamic acid has been rearranged to give a 20 per cent yield of salicylanilide (26).

Substituted salicylanilides have also been prepared by the direct action of reagents upon salicylanilide. Thus, nitration (16), chlorination with sulfuryl chloride (27), sulfonation (28), and bromination (14, 29, 30) of salicylanilide or substituted salicylanilides have been studied.

The selection of the type of compounds prepared in the present work was partly based on results with nitro- (31, 32) and halo-2-furanacrylanilides (32), which exhibit bacteriostatic activity, and 4′-chloro-5-nitro-2-furanacrylanilide (33), which exhibits anthelmintic activity. Thus it seemed that halonitrosalicylanilides, which would have a number of chemical features common to some of the above mentioned 2-furanacrylanilides and also possess the salicylanilide moiety of known antifungal activity, might exhibit broader antimicrobial action than either of the above materials. Indeed, when 4′-chloro-5-nitrosalicylanilide was prepared and tested, this was found to be the case. Therefore, seventeen analogs of this compound were prepared to determine the effects, due to changes in the positions and the amounts of various groups present or due to the kinds of halogen substituent, upon antimicrobial activity.

Experiments have been carried out to prepare these compounds by three methods. Reaction of the respective dinitro- or nitrosalicoyl chlorides with an excess of the halogenated aniline in benzene was found to be a satisfactory method which gave the halonitrosalicylanilides in 71 to 93 per cent yields. Most of the salicylanilides in the present work were made by this method. However, interaction of the acid, the aniline, and phosphorus trichloride in benzene was also found to be useful. In a third method, a Schotten-Baumann reaction between the acid chloride and the aniline in the presence of aqueous sodium hydroxide was performed to give, however, low yields of the desired anilides highly contaminated by the acids. The compounds prepared and their physical properties are given in Table I. The known materials 4′-chlorosalicylanilide and 3-nitrosalicylanilide were also prepared, using the Schotten-Baumann method, for comparison with the other anilides in the biological testing.

The 3- and 5-nitrosalicylic acids used in this work were obtained by a low temperature (0°) nitration of salicylic acid which yielded an isomeric mixture of about 3:1 of the 5- and 3-nitro acids. Separation was effected by taking advantage of the difference in solubility of the potassium salts of these acids (34).

The acid chlorides were than prepared directly from the above acids upon refluxing with thionyl chloride in benzene in the presence of a small amount of anhydrous aluminum chloride. Three hours were required for the completion of this reaction with the 3-nitrosalicylic acid. However, sixteen hours were required for the 5-nitro- and 3,5-dinitrosalicylic acids to form the chlorides. The aluminum chloride catalyst was important for the latter two acids since without it the reaction was not complete in sixteen hours. 5-Nitrosalicoyl chloride had not been previously prepared.

The halonitrosalicylanilides prepared in this work are insoluble in water. However, because of the presence of the phenolic group whose acidity is enhanced by the nitro group on the same ring, aqueous solutions of about 0.05 per cent could be prepared for antimicrobial tests by adding an equivalent of suitable base such as sodium hydroxide to the water.

PHARMACOLOGICAL ACTIVITY

Antibacterial screening by the agar cup-plate method among all of the compounds listed in Table I indicated that the halo-3,5-dinitrosalicylanilides had little or no activity against the bacteria referred to below. However, the halo-3-nitro- and halo-5-nitrosalicylanilides are potent antimicrobials against a number of bacteria and fungi. Preliminary comparison tests by the agar cup-plate method among the salicylanilides indicated that all of the halo-3-nitrosalicylanilides had very similar potencies and specificities of action regardless of the number, kind, or position of the halogens in the aniline ring. This was also found to be true for the halo-5-nitrosalicylanilides. Therefore, 4′-chloro-3-nitrosalicylanilide (I) and 4′-chloro-5-nitrosalicylanilide (II) were selected as representatives of these two groups for further testing. The influence of the various substituent groups particularly against *Micrococcus pyogenes* var. *aureus* is illustrated in Table II. I also showed a threshold inhibition at 18 mcg./cc. against *Micrococcus flavus* and II showed inhibition at 10 mcg./cc. against *Alcaligenes faecalis*.

These results indicate that both the nitro and halogen groups confer antibacterial activity upon the salicylanilide parent molecule, which when unsubstituted exhibits very little antibacterial action. The absence of much activity among either the halogenated anilines or the nitrosalicylic acids indicates that this modification in activity of salicylanilide is not due simply to the fact that the molecule is now a combination of an aryl nitrophenol and a halogenated aniline. Instead, it appears to be a more complex gross effect requiring, particularly, the anilide linkage to complete the

TABLE I.—HALONITROSALICYLANILIDES

Compound	M. p.,[a] °C.	Formula	Analysis—Carbon, %—Calcd.	Carbon, %—Found	Hydrogen, %—Calcd.	Hydrogen, %—Found
4′-Fluoro-3-nitrosalicylanilide	141.5–142	$C_{13}H_8FN_2O_4$	56.53	57.19	3.20	3.35
2′-Chloro-3-nitrosalicylanilide	171.5–172.5	$C_{13}H_9ClN_2O_4$	53.34	53.42	3.09	3.16
3′-Chloro-3-nitrosalicylanilide	152.5–153.5	$C_{13}H_9ClN_2O_4$	53.34	53.36	3.09	3.06
4′-Chloro-3-nitrosalicylanilide	154–156	$C_{13}H_9ClN_2O_4$	53.34	53.10	3.09	2.99
4′-Bromo-3-nitrosalicylanilide	158–158.5	$C_{13}H_9BrN_2O_4$	46.31	46.36	2.69	2.69
4′-Iodo-3-nitrosalicylanilide	175–178	$C_{13}H_9IN_2O_4$	40.64	41.26	2.36	2.67
2′,4′-Dichloro-3-nitrosalicylanilide	241	$C_{13}H_8Cl_2N_2O_4$	47.71	47.42	2.46	2.28
4′-Fluoro-5-nitrosalicylanilide	240	$C_{13}H_8FN_2O_4$	56.53	58.32	3.20	3.37
2′-Chloro-5-nitrosalicylanilide	200–203	$C_{13}H_9ClN_2O_4$	53.34	53.11	3.09	3.01
3′-Chloro-5-nitrosalicylanilide	230	$C_{13}H_9ClN_2O_4$	53.34	53.55	3.09	3.07
4′-Chloro-5-nitrosalicylanilide	252.5	$C_{13}H_9ClN_2O_4$	53.34	53.51	3.09	3.09
4′-Bromo-5-nitrosalicylanilide	242–245	$C_{13}H_9BrN_2O_4$	46.31	46.15	2.69	2.49
4′-Iodo-5-nitrosalicylanilide	261–264	$C_{13}H_9IN_2O_4$	40.64	41.05	2.36	2.56
2′,4′-Dichloro-5-nitrosalicylanilide	261–262	$C_{13}H_8Cl_2N_2O_4$	47.71	47.65	2.46	2.20
2′,4′,6′-Trichloro-5-nitrosalicylanilide	233.5–234	$C_{13}H_7Cl_3N_2O_4$	43.28	43.09	1.95	1.81
2′-Chloro-3,5-dinitrosalicylanilide	216.5–218	$C_{13}H_8ClN_3O_6$	46.24	45.96	2.39	2.28
3′-Chloro-3,5-dinitrosalicylanilide	211–212	$C_{13}H_8ClN_3O_6$	46.24	47.67	2.39	2.81
4′-Bromo-3,5-dinitrosalicylanilide	227	$C_{13}H_8BrN_3O_6$	40.86	41.09	2.11	2.46

[a] Corrected.

TABLE II.—ANTIBACTERIAL ACTIVITIES

Compound	Bacterial Inhibition Threshold Concentrations mcg./cc. *S. aureus*[a]	*B. subtilis*[b]	*E. coli*[c]
5-Nitrosalicylic acid	500[d]	..	..
3,5-Dinitrosalicylic acid	500	..	..
4-Chloroaniline hydrochloride	500	..	..
2,4-Dichloroaniline hydrochloride	500[d]	..	..
Salicylanilide	1000[d]	1,000[d]	1,000[d]
4′-Chlorosalicylanilide	10	..	..
3-Nitrosalicylanilide	10	..	..
4′-Chloro-3-nitrosalicylanilide	1	1	1,000[d]
4′-Chloro-5-nitrosalicylanilide	1	0.3	1,000[d]

[a] *Micrococcus pyogenes*, var. *aureus*.
[b] *Bacillus subtilis*.
[c] *Escherichia coli*.
[d] No inhibition.

TABLE III.—SERIAL BROTH DILUTION DETERMINATIONS

Organism	Inhibiting Dose, μg./cc. 4′-Chloro-3-nitrosalicylanilide	4′-Chloro-5-nitrosalicylanilide
Micrococcus pyogenes var. *aureus*	4–8	1–2
Bacillus subtilis	9–10	1–2
Pseudomonas aeruginosa	>200	>200
Escherichia coli	>200	>200
Alcaligenes faecalis	80–100	20–40
Proteus (species unknown)	>200	60–80

combination of moieties for the manifestation of markedly increased antibacterial activity.

I and II were compared and examined more thoroughly in broth serial dilution tests. The results of these tests are shown in Table III. From the screening tests and from the serial dilution tests it can be seen that the 5-nitro derivative is consistently more potent than the 3-nitro compound. Furthermore, it is evident that these halonitrosalicylanilides are consistently active against the Gram-positive organisms but are often inactive or less active against the Gram-negative organisms.

Upon testing the analogs against fungi a greater diversity of action was exhibited than against the bacteria. The 3,5-dinitrosalicylanilides again showed none or very little antimicrobial action. In these tests, I showed decidedly more inhibiting activity than II against *Trichophyton mentagrophytes*, *Candida albicans*, and *Penicillium luteum*. From general comparison tests carried out against these organisms, I and 3′-chloro-3-nitrosalicylanilide (III) exhibited considerably greater potencies against the above organisms than any of the other compounds. Inhibition tests at 286 p. p. m. in agar were carried out. At this level I and III were able to inhibit all growth of the *T. mentagrophytes* at fourteen days. However, for *P. luteum*, although complete inhibition was not obtained, these compounds caused the lowest radial growth rate. Further tests with III showed it to inhibit completely *E. floccosum* at 7.1 p. p. m. at twenty-five days. Upon comparing I and salicylanilide in a twenty-seven-day serial dilution test against *C. albicans*, both materials gave similar inhibition threshold values at 45 p. p. m.

The last results, together with some of the other numerical threshold values, indicate that although the nitro and halogen substituents confer antibacterial properties upon salicylanilide, they neither enhance nor lower its already high antifungal activity.

Preliminary toxicity studies indicate that the halo-3-nitrosalicylanilides are consistently at least several times more toxic to rats than the 5-nitro isomers. For example, I was found to have an intraperitoneal LD_{50} of 34 mg./Kg. in rats, while II had an LD_{50} of over 125 mg./Kg. in the same determinations.

EXPERIMENTAL

3- and 5-Nitrosalicylic Acids.—Nitrations in water, acetone, and acetic acid were carried out. However, the most satisfactory method was to combine a low temperature nitration procedure in sulfuric acid with the principle of the separation procedure of Meldrum and Hirve (34). Thus, 2,000 Gm. (14.5 mole) of salicylic acid was dissolved in 4 L. of concentrated sulfuric acid and the solution was cooled with dry ice–alcohol to 0°. Then with rapid stirring and continued cooling a precooled mixture of 916 ml. of concentrated nitric acid and 916 ml. of concentrated sulfuric acid was added, keeping the temperature below 7°. One hour was required for addition, after which time the mixture was poured into 24 L. of water, allowed to stand overnight, filtered by vacuum, washed, and dried at 70° for sixteen hours. A yield of 2,550 Gm. (96.0% yield) of the cream colored isomeric mixture was obtained. One hundred and sixty-three Gm. (0.89 mole) of the mixture was dissolved with stirring in 3,300 ml. of boiling water containing 114.0 Gm. of potassium carbonate and 15 Gm. of charcoal. The hot solution was filtered and allowed to stand at room temperature overnight. The orange crystals of the potassium salt of 3-nitrosalicylic acid which had precipitated were filtered by vacuum and washed three times with cold water. They were then dissolved in 1 L. of 90° water, the solution was filtered, and 60 ml. of 35.0% sulfuric acid was added to the hot filtrate with vigorous stirring to give an immediate precipitate of 3-nitrosalicylic acid. The solution was stirred and cooled to room temperature (about three hours) and the white solid that separated was removed by vacuum filtration and washed with water. This product was dried at 100° for sixteen hours to give 37.5 Gm. (22.5% yield) of 3-nitrosalicylic acid, m. p. 147.5–148° (lit. (35) m. p. 146–148°), which now had a canary yellow color. Upon acidifying the original filtrate and reducing its volume to one-fifth, 90 Gm. (55.0% yield) of 5-nitrosalicylic acid, m. p. 234–236° (lit. (35) m. p. 234°) was obtained upon filtering, washing, and drying.

3-Nitrosalicoyl Chloride.—Eighty Gm. (0.44 mole) of 3-nitrosalicylic acid, 52.0 ml. of thionyl chloride, and 320 ml. of benzene were refluxed for two hours. The solution was filtered and the solvents removed by evacuating at room temperature for sixteen hours. A yield of 82.6 Gm. (93.6%) of yellow crystals of 3-nitrosalicoyl chloride, m. p. 55.5–57.5° (lit. (36) m. p. 59–61°), was obtained.

Anal.—Calcd. for $C_7H_4ClNO_4$: C, 41.70; H, 2.00. Found: C, 43.62; H, 2.04.

When the acid chloride was heated to 60° during the removal of the solvents, hydrogen chloride was given off and the resultant product had a much poorer melting point.

5-Nitrosalicoyl Chloride.—This was prepared by essentially the same procedure used above, however, it was found that the reaction proceeded much more slowly. Thus, without a catalyst, at the end of sixteen hours, a considerable amount of the benzene-insoluble 5-nitrosalicylic acid had remained unconverted to the benzene-soluble acid chloride. When a small amount of anhydrous aluminum chloride was added to the reaction mixture, no insoluble material remained after sixteen hours of refluxing. Thus, 50.0 Gm. (0.27 mole) of 5-nitrosalicylic acid, 200 ml. of benzene, 30.0 ml. of redistilled thionyl chloride, and 0.1 Gm. of anhydrous aluminum chloride gave 49.7 Gm. (91.6% yield) of crude 5-nitrosalicoyl chloride. It was found that if heat was applied (60°) while removing last traces of solvents from the residue, hydrogen chloride was evolved and the syrupy residue would not solidify. If heat was not applied the crude acid chloride was obtained as a hard brittle glass. A portion of this glass was recrystallized from 1:1 petroleum ether–benzene to give, in the second crop, a pure sample of 5-nitrosalicoyl chloride, m. p. 78–80°, which was a cream colored amorphous solid.

Anal.—Calcd. for $C_7H_4ClNO_4$: C, 41.70; H, 2.00. Found: C, 42.59; H, 2.10.

3,5-Dinitrosalicoyl Chloride.—This material had been previously prepared with phosphorus pentachloride (24, 37). In the present work, the same method as was used above to prepare the 5-nitrosalicoyl chloride was applied. Here again sixteen hours of refluxing was necessary to complete the reaction with the aid of the anhydrous aluminum chloride catalyst. The crude 3,5-dinitrosalicoyl chloride was used in the preparation of the salicylanilides.

Halonitrosalicylanilides, 4′-Chloro-3-nitrosalicylanilide.—The method described below represents the general procedure used to prepare most of the halonitrosalicylanilides under discussion. When 3-nitrosalicoyl chloride was added to pyridine an exothermic reaction took place to give a pyridine-insoluble product. Thus, it was not possible to use pyridine as a solvent for anilide formation. A solution of 25 Gm. (0.12 mole) of 3-nitrosalicoyl chloride in 200 ml. of benzene was added with shaking to 50 Gm. (0.39 mole) of *p*-chloroaniline in 150 ml. of benzene. An immediate yellow precipitate formed and the reaction mixture was allowed to stand overnight and vacuum filtered. The solid obtained was washed with small amounts of benzene, air dried, pulverized, and then stirred for thirty minutes in 100 ml. of 10% hydrochloric acid. The product was vacuum filtered, washed well with water, and dried at 90° for sixteen hours to give 28.5 Gm. (78.5% yield) of crude 4′-chloro-3-nitrosalicylanilide, m. p. 153–156°. This was crystallized from 600 ml. of ethanol to give 23.0 Gm. of yellow needles in a first crop and 3.6 Gm. more when the alcohol filtrate was reduced to one-sixth. Thus, a total of 26.6 Gm. of 4′-chloro-3-nitrosalicylanilide, m. p. 154–156° was obtained.

Anal.—Calcd. for $C_{13}H_9ClN_2O_4$: C, 53.34; H, 3.09. Found: C, 53.10; H, 2.99.

When 2,4-dichloroaniline was reacted with the nitrosalicoyl chlorides, the rate of reaction was slower than with the monohaloanilines and a precipitate did not start to form until about one hour after mixing and did not fill the mixture until several hours later. With the monohaloanilines, precipitation occurred immediately and satisfactory yields could be obtained in about one hour after mixing. With 2,4,6-trichloroaniline and 3-nitrosalicoyl chloride, the anilide did not form until after five days. However, with 5-nitrosalicoyl chloride and the trichloroaniline the salicylanilide was obtained only after seven days standing. The halo-3-nitro and halo-3,5-dinitrosalicylanilides were crystallized from ethanol, while the

5-nitro compounds were crystallized from ethanol or ethanol-dimethylformamide.

4′-Chloro-5-nitrosalicylanilide by the "Phosphorus Trichloride Method."—Four grams (0.022 mole) of 5-nitrosalicylic acid, and 6 Gm. (0.047 mole) of *p*-chloroaniline were ground, intimately mixed, and placed into 35 ml. of benzene containing 1.5 ml. of phosphorus trichloride. The mixture was refluxed for sixteen hours, cooled, and filtered. The solid was crushed and suspended in 10% hydrochloric acid, filtered, and crystallized from ethanol to give 5.4 Gm. (83.1% yield) of 4′-chloro-5-nitrosalicylanilide, m. p. 249–250°. Upon admixture with 4′-chloro-5-nitrosalicylanilide prepared from the acid chloride (m. p. 252.5°) no depression of melting point occurred.

4′-Chlorosalicylanilide.—This was prepared by the Schotten-Baumann procedure. Salicoyl chloride was prepared according to the method of Wolffenstein (23) from thionyl chloride and salicylic acid. Thus 20.0 Gm. (0.13 mole) of salicoyl chloride in 20 ml. of benzene was added to a rapidly stirred mixture of 16.4 Gm. (0.13 mole) of *p*-chloroaniline and 15 Gm. of sodium hydroxide in 100 ml. of water. The acid chloride was added over a ten-minute period and the mixture was then stirred for thirty minutes. No solid had separated and thus the aqueous layer was acidified to pH 6 whereby 15.0 Gm. of solid that started to melt at 134° was obtained. Further acidification of the aqueous layer yielded salicylic acid, m. p. 157–159° (lit. (38) m. p. 157–159°). The solid first obtained was crystallized from ethanol to give 4.3 Gm. (13.4% yield) of 4′-chlorosalicylanilide, m. p. 169–170° (lit. (39) m. p. 168–169°).

3-Nitrosalicylanilide.—This was also prepared by the Schotten-Baumann method. In this preparation the product precipitated and was filtered directly from the reaction mixture. Acidification of the aqueous phase yielded only a small amount of additional material. Thus 4.0 Gm. (0.02 mole) of 3-nitrosalicoyl chloride and 1.9 Gm. (0.02 mole) of aniline in benzene with 1.5 Gm. of sodium hydroxide in 20 ml. of water gave 3.5 Gm. of insoluble precipitate. One gram of this material was suspended in boiling 4% sodium carbonate to remove any 3-nitrosalicylic acid and then suspended in 10% hydrochloric acid to remove unused aniline. The solid was filtered, washed, and recrystallized from ethanol to give 0.4 Gm. (25.0% yield for the total of original product assuming 40% recovery on purification) of 3-nitrosalicylanilide, m. p. 124–125° (lit. (9) m. p. 121–122°).

Antimicrobial Screening.—All of the compounds were tested in aqueous solutions made by dissolving, with warming, up to several hundred milligrams in 100 ml of water containing an equivalent or a slight excess of sodium hydroxide, to give solutions of pH 7 to 8. Antibacterial screening tests were done by the agar-cup plate method, placing the test compound solutions into the cups and seeding the agar with the organism under study (40). Values of threshold concentrations were approximated either by means of inoculated serial dilutions of the compounds or by plotting the logarithms of the concentrations against the zones of inhibition for several concentrations and extrapolating to the threshold values.

The broth dilutions were prepared by incorporating appropriate graded amounts of the compounds in Difco Yeast Beef Broth which was maintained at a uniform concentration throughout each series. The tubes of the various series were inoculated with a loopful of inoculum from twenty-four hour cultures of the test organisms grown in the same medium. Incubation for forty-eight to seventy-two hours was usually sufficient although all were observed during a period of one week.

Fungistatic activity was determined by inoculating the test organism into a medium composed of a base of plain agar containing the test compound overlaid by a layer of potato dextrose agar. From periodic measurements of the radii of the colonies, the inverse of the radial growth rate in mm. per day was considered an index of the fungistatic efficacy of the test substance.

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Thermosterilized Acriflavine Emulsion Ointment*

By E. MENCZEL and S. GRUN

Four thermostable o/w emulsion bases were selected for the preparation of acriflavine ointments with which absorbent gauze was impregnated and steam sterilized for topical antisepsis. Bacteriostatic tests indicated that at 1 per cent w/w concentration of acriflavine effective antiseptic ointments were produced.

VARIOUS ACRIFLAVINE OINTMENTS are clinically used for topical antisepsis, without the complications of resistance encountered with antibiotics and other oral or parenteral bacteriostatic agents. Experimental data indicate that the bacteriostatic activity of acriflavine ointments depends also upon the ointment bases. Frank and Stark (1) tested the effectiveness of acriflavine[1] incorporated in a water-in-oil emulsion ointment base containing cholesterol; even at the concentration of 1.5 per cent w/w acriflavine, the growth of *Staphylococcus aureus* and *E. coli* was not inhibited. Owing to anionic cationic interaction, oil-in-water emulsion ointments of acriflavine with aqueous cream (3) B. P. base[2] could not have been prepared. Similarly, a sodium alginate base was not found suitable. Unguentum glycerini, a greaseless starch glycerite base official in the Swiss Pharmacopeia (4), did not hinder the antiseptic activity of the acriflavine incorporated, at the range of 1–1.5 per cent w/w concentration. This is in accordance with the prevailing concepts that some antiseptic ointments are more effective in water-miscible bases having a high water content (5). However, starch glycerite is apt to deteriorate rapidly; furthermore, water-miscible bases devoid of an oily phase are considered to be drying and not applicable to both dry thin and thick fatty skins (6). Eventually, a stable water-miscible emulsion base of high water content appears to be the formulation of choice for an acriflavine ointment. The clinical use of an acriflavine ointment in surgical theater and wards in the treatment of burns and other topical infections warrants absolute sterility to avoid reinfection by resistant microorganisms. Sterilization could be performed by one of the thermal procedures which, however, might prove deleterious to the thermolabile emulsion system. Benerito and Singleton (7) and Lambert, *et al.* (8), have reported studies on the preparation of thermostable emulsions for parenteral use which could be sterilized. Thermosterilizability will, therefore, feature as a prime factor in evaluating the o/w emulsion bases for an effective acriflavine ointment. As far as consistency is concerned, it should be softer than ordinary ointment bases and this might enable ready incorporation into a medicated dressing.

EXPERIMENTAL

Emulsifying Agents.—The preparation of o/w emulsion ointments of acriflavine precludes the use of anionic emulsifying agents. The selection has, therefore, to be made from the limited list of nonallergic, nonirritating cationic and nonionic emulsifying-agent agents. As to the thermosterilizability of an o/w emulsion produced, a certain clue can be obtained by ascertaining the inversion temperature of a 1% solution of the emulsifying agent. The procedure of the test was exactly the same as that suggested by Benerito and Singleton (7). The nonionic emulsifying agents, polysorbate 80 and polyoxyl 30 stearate, were found to have high temperature inversion points[3] and eventually these emulsifying agents might produce thermostable o/w emulsion ointments. Preference was given to polyoxyl 30 stearate over its homolog containing 40 polyethylene groups (official in U. S. P. XV) owing to its high degree of diffusibility (9) which cannot be over-emphasized in this type of preparation. The nonionic emulsifying agents, trademarked in Germany as Cremophors, proved to have certain merits (10, 11) in emulsion technology but their thermostability could not have been deducted from the Benerito-Singleton test. A highly purified grade of egg lecithin (cationic emulsifying agent) deteriorated upon temperature rise when applying the Benerito-Singleton test.

Preparation of the o/w Emulsion Ointments, Stability Controls, and Their Physical Properties.—Acriflavine o/w emulsion ointments were formulated to contain 20% petrolatum and/or liquid petrolatum, the water content adjusted to exceed 50%. It was expected that such preparations will be creamlike soft (even pourable) and could be incorporated into a medicated dressing. The procedure used in the preparation of the test ointments was according to the conventional pharmacopeial method as follows:

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[1] The grade official in the "British Pharmacopoeia 1948" which consists of a mixture of diaminoacridine hydrochloride, diaminoacridine dihydrochloride, and hydrochloride of diaminomethyl acridinium chloride; this is not equivalent to the acriflavine official in the "British Pharmaceutical Codex 1954" which, too, differs from acriflavine and acriflavine hydrochloride official in the "National Formulary X" (2). All these derivatives of aminoacridine bear cationic properties.

[2] Aqueous cream (simple cream) B. P. (3) consists of 30% emulsifying ointment B. P. containing anionic sodium lauryl sulfate as the main emulsifying agent.

[3] Inversion point is defined as the temperature at which 1% aqueous solution of the emulsifying agent becomes turbid; inversion temperature of polysorbate 80 is at 85°, whereas that of polyoxyl 30 stearate is at 95°. Mention should be made that with the anionic sodium lauryl sulfate a clear solution is obtained even at 100°.

The oily phase including the emulsifying agent (wherever applicable) was melted on a water bath. The aqueous phase containing acriflavine[4] and the emulsifying agent (wherever applicable) was warmed to the same temperature; both phases were mixed continuously until congealing. The stability controls were conducted stepwise starting with visual observations for any physical changes on storage at room temperature; controls were made at logarithmic intervals starting from 10 seconds and onwards, 100 seconds, 1,000 seconds, 10,000 seconds, 10^5 seconds, 10^6 seconds (11 days, 13 hours, 46 minutes, 40 seconds).[5] Any ointment in which could be detected a perceptible separated oily or aqueous layer was discarded. This was followed by incubation test for 10^6 seconds at 37° (±2°) through which the ointment had to preserve its stability. The last stability test was conducted upon autoclaving (15 lb./inch^2 pressure, 121°, 10^3 seconds = 16 minutes, 40 seconds). Ointments which failed to be re-emulsified to proper consistency, were considered inadequate. The three types of stability controls combined proved valuable in selecting the emulsion bases for acriflavine ointments. Preliminary tests have indicated that the storage tests at 37°, which were advocated by Husa and co-workers (12), will not suffice for the evaluation of a thermostable emulsion. Autoclaving stability tests of emulsions were suggested for nonpharmaceutical emulsions (13) and were employed too in a study of parenteral thermostable fat emulsions (8). The results of our study confirm that thermostability is best ascertained by the autoclaving test.

The emulsion ointments of acriflavine which passed the rigid stability tests were subjected to further physical tests: type of emulsion, melting point, and viscosity.

The o/w type of the emulsion ointments was ascertained by the dye method and water miscibility (14). Melting point was determined by the official B. P. method for fats which was somewhat adapted. Apparent viscosities of the emulsion ointments were determined by three different methods: (*a*) rheological falling sphere method at several temperatures; (*b*) Scott orifice efflux viscometer at the fluid phase above melting temperature with and without pressure; and (*c*) Brookfield Synchro-Lectric rotational viscometer at varying rates of shear and at a wide temperature range.

Sterilization of the Emulsion Ointments.—The petrolatum used in the preparation of "petrolatum gauze" is sterilized at 165–170° for 2 hours according to the official directions of the U. S. P. (18). Oven sterilization at the same temperature for 2.5 hours (15, 17) at least was found reliable for gauze dressings impregnated with petrolatum, oils, and fatty ointments. These sterilization procedures involved a very high temperature for a rather long period which might prove detrimental to the emulsion ointments and their constituents. Emulsion ointments cannot be sterilized by bacterial filtration at their melting temperature since the filtering pores are smaller in diameter than that of most of the internal phase globules. However, the presence of water in the selected emulsion ointment enables steam sterilization (16) at 15 lb./inch^2 (121°) for 30 minutes; as long as saturated steam is used at its phase boundary (19) this less drastic method is highly safe. Furthermore, this method of sterilization coincided with the rigid autoclaving tests and therefore the selected emulsion ointments proved to be thermosterilizable. The rate of redispersion after autoclaving was not equal for all emulsion ointments but in all cases it has been secured even after repeated sterilizations.

Bacteriostatic Tests.—The antiseptic activity of the acriflavine ointments was evaluated by the bacterial inhibition test (20, 21) which was modified to secure most concordant results. The standard agar cup plate method utilizing *Staphylococcus aureus* was expanded and the ointments were tested similarly with virulent strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes;* since these too appear quite often in skin infections. The ointments were spread in uniform strips of dimensions 50 × 6 mm. on sterile agar plates which were inoculated serially with $^1/_{10}$ and $^1/_{100}$ dilutions of the bacterial culture, and incubated for 24 to 48 hours. In the case of *Streptococcus pyogenes*, controls were made with sterile blood agar plates. Inhibition zones were measured in millimeters from one side of the ointment strip. Acriflavine was incorporated in the selected ointment bases in ascending amounts starting with 0.1% w/w up to 2% w/w; considering the average molecular weight of acriflavine as 281.4, the concentrations were converted into millimole % (m*M* %) and accordingly the range extended between 0.355 m*M* % and 7.10 m*M* %.

Emulsion Bases.—Single and compound formulations with polysorbate 80 did not lead to stable ointment bases. The incorporation of cetostearyl alcohol did not induce stability at 37°. The results with polysorbate 80 did not coincide with that indicated by Benerito-Singleton tests: in spite of the high inversion point of the water solubility of polysorbate 80, autoclavable emulsion ointments could not be prepared with it.

The formulations with Cremophor A fest (solid) combined with Cremophor A flussig (liquid) were not stable at 37°. On the other hand Cremophor A solid as a single emulsifying agent produced an emulsion base stable at 37° but failed at steam sterilization temperature. Cremophor Ap solid combined with Cremophor A solid as recommended by the manufacturer (11) led to a base unstable at 37°.

Preliminary formulations with polyoxyethylene 30 stearate as a single o/w emulsifying agent (even up to 10%) were unsuccessful. Omitting the petrolatum and/or liquid petrolatum and upon incorporation of cetostearyl alcohol (10–25%) and propylene glycol (10–12%) smooth ointments were obtained which, however, were too dry. On further trials, following the U. S. P. XV formula for hydrophilic ointment, we have found that with cetostearyl alcohol 3% w/w, and polyoxyethylene 30 stearate 3% w/w, a suitable thermosterilizable emulsion ointment could be produced (base I). The substitution of cetostearyl alcohol with Cremophor A solid in this base led to a higher viscosity. Screening the many bases of this kind prepared, it was inferred that the most appropriate emulsion base consisted of a blend of polyoxyethylene 30 stearate 5% w/w and Cremophor A solid 5% w/w (base II). Further experimentation indicated that far more stability could be

[4] B. P. 48 grade was used throughout this study.

[5] The theoretical and practical aspects of logarithmic periodical emulsion controls is to be reported in a further communication apart from this study.

achieved (redispersion after autoclaving unnecessary) by adding cetostearyl alcohol to the constituents of base II. The best formulation included cetostearyl alcohol 2.5% w/w, polyoxyethylene 30 stearate 2.5% w/w, and Cremophor A solid 4% w/w (base III).

The reduction of the water content to about 50% without increasing the concentration of the oleaginous phase can be effected by incorporating water-soluble or water-miscible constituents. Polyethylene glycols of molecular weights 400 and 4000, respectively, proved valuable in official ointment formulations but alone are considered to produce improper ointments (6). The emulsification of aliphatic oleaginous ingredients in the presence of polyethylene glycols proceeds rather with some difficulty, and even then the best emulsifying agent was found to be polyoxyethylene 30 stearate. Upon autoclaving some of polyethylene glycol 4000 crystallized out and this had to be replaced by various other polyethylene glycols. Good results were obtained by decreasing the proportion of polyethylene glycol 400 and incorporating polyethylene glycol 900 and 1500, respectively (base IV).

The results with polyoxyethylene 30 stearate confirmed the Benerito-Singleton test for the selection of the main emulsifying agent producing thermostable emulsions.

The consistency of the lecithin o/w emulsion bases were appropriate but proved to be unstable, particularly upon autoclaving; this could have been predicted from the Benerito-Singleton test.

The composition and the physical characteristics, including the stability data of the four selected bases, are listed in Table I. It should be noted that the o/w emulsion type offers washability and that the decline in apparent viscosities of these bases at body temperature enables proper diffusion, spreading, and penetration. The rheograms of these bases at 20° reproduced in Figs. 1 and 2 indicate plastic viscosities with thixotropic features—particularly of bases I and II; owing to the different range of viscosities two separate spindles were used: Spindle II (Brookfield Synchro-Lectric viscometer model HBT) for bases I and II and spindle III for bases III and IV.[6] The rheogram of glycerin (99% w/w) which possesses Newtonian viscosity was also included in Figs. 1 and 2 for comparison.

Antibacterial Effectiveness.—In spite of the oleaginous phase in the selected o/w emulsion bases the antibacterial activity of the incorporated acriflavine was not hindered as compared with that of a greaseless starch glycerite base (see Table II). It was reascertained that the optimal antiseptic concentration of acriflavine in effective ointments bases is about 1% w/w (3.55 m*M* % w/w). Equivalent concentrations of Flavazole (a proprietary equimolecular combination of 2,8-diaminoacridine and sulfathiazole) in one of the selected ointment bases did not possess superior antiseptic activity. Furthermore, the incorporation of 1% sulfathiazole into 1% acriflavine o/w emulsion ointment did not enhance the antibacterial activity. On the other hand, cream of proflavine B. P. C. 1954 (22) which is a w/o emulsion containing only 0.1% proflavine did not inhibit the growth of any of the test organisms.

[6] The thixotropy of bases III and IV is less pronounced and appears only at the lowest r. p. m. (see Fig. 2, base of slope C).

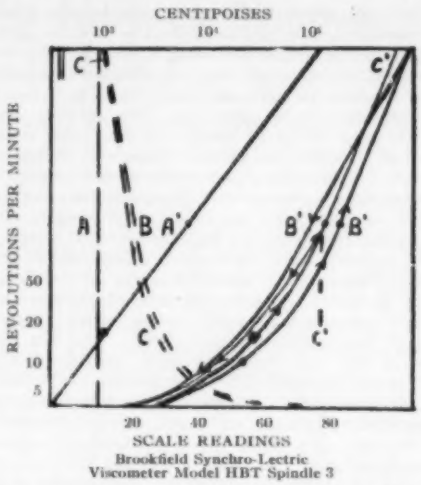

Fig. 1.—Rheogram of bases I and II: Shearing stress (scale readings) and apparent viscosity (centipoises—log scale) *versus* rate of shear (revolutions per minute). ⟷ Slope of shearing stress upon increasing or decreasing rate of shear. - - - - - - - Slope of apparent viscosity upon increasing rate of shear. A, A′, glycerin 99% w/w. B, B′, base I. C, C′, base II.

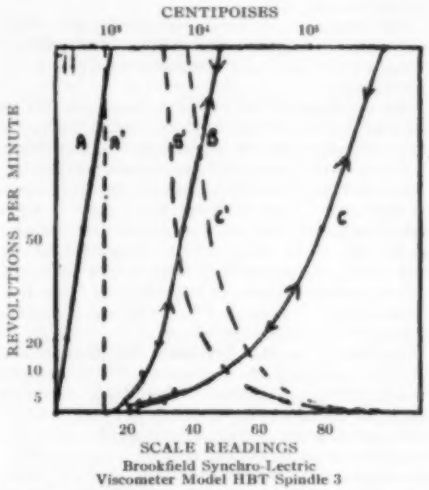

Fig. 2.—Rheogram of bases III and IV: shearing stress (scale readings) and apparent viscosity (centipoises—log scale) *versus* rate of shear (revolutions per minute). ⟷ Slope of shearing stress upon increasing or decreasing rate of shear. - - - - - - - Slope of apparent viscosity upon increasing rate of shear. A, A′, glycerin 99% w/w. B, B′, base III. C, C′, base IV.

Preparation of Acriflavine Antiseptic Dressings.—Absorbent gauze is cut in strips and folded according to the pharmacopeial directions for petrolatum gauze (17) and filled into heat-sterilizable wide-mouth

TABLE I.—PHYSICAL CHARACTERISTICS OF ACRIFLAVINE OINTMENTS 1% w/w (3.55 mM. %) WITH THE SELECTED EMULSION BASES

Constituents of the Bases	Base I, %	Base II, %	Base III, %	Base IV, %
Nonaqueous phase (oleaginous)	20 w/w	20 w/w	20 w/w	20 w/w
Petrolatum	...	20 w/w	...	...
Liquid petrolatum	20 w/w	...	20 w/w	20 w/w
Aqueous phase	74 w/w	70 w/w	71 w/w	74 w/w
Water content	64 w/w	60 v/w	61 v/w	47 v/w
Propylene glycol	10 w/w	10 w/w	10 w/w	10 w/w
Polyethylene glycol 400	...	...	...	3 w/w
Polyethylene glycol 900	...	...	...	4 w/w
Polyethylene glycol 1500	...	...	...	10 w/w
Emulsifying agents	6 w/w	10 w/w	9 w/w	6 w/w
Cetostearyl alcohol	3 w/w	...	2.5 w/w	3 w/w
Cremophor A solid	...	5 w/w	4 w/w	...
Polyoxyl 30 stearate	3 w/w	5 w/w	2.5 w/w	3 w/w
Consistency	Viscous cream-like (pourable)	Viscous cream-like (pourable)	Soft semisolid (nonpourable)	Very viscous cream-like (pourable)
Melting point	−4°	+1°	44°	−10°
Specific gravity	0.982 (30°)	0.978 (35°)	0.966 (40°)	1.012 (36°)
Type of emulsion	o/w	o/w	o/w	o/w
Stability at 10^6 seconds at room temperature	Stable	Stable	Stable	Stable
Stability at 10^6 seconds at "incubation" 37° (±2°)	Stable	Stable	Stable	Stable
Stability at 10^3 seconds at 121°, 15 lb./inch² (autoclaving)	Stable (redispersion necessary)	Stable (redispersion necessary)	Stable	Stable (redispersion necessary)
Stability at sterilization (121°, 15 lb./inch², 30 min.) as a medicated dressing (gauze impregnated with acriflavine emulsion ointment)	Very slight separation of emulsion phases redispersed by one or two "shakes"	Slight separation of emulsion phases, redispersion effected after intermittent shaking	Most stable preparation (redispersion not necessary)	Separated emulsion phases redispersed after continuous shaking up to congelation
Apparent viscosity decline with increasing temperature	Centipoises	Centipoises[g]	Centipoises	Centipoises
10°	83,200[a]	62,000[b]	208,000[b]	196,800[b]
15°	65,600[a]	58,400[a]	172,000[b]	192,000[b]
20°	51,200[a]	44,800[a]	128,000[b]	188,000[b]
25°	44,000[a]	24,800[a]	112,000[b]	152,000[b]
30°	45,600[a]	22,400[a]	100,000[b]	110,000[b]
30°	30,300[d]	...	...	...
35°	32,000[a]	20,800[a]	100,000[b]	68,000[b]
35°	...	14,700[d]	...	...
36°	...	...	...	15,100[d]
40°	16,800[a]	4,000[c]	84,000[b]	4,000[c]
40°	...	...	9,800[d]	...
50°	6.07[e]	6.95[e]	6.61[e]	9.26[e]
50°	2.85[f]	4.22[f]	4.17[f]	4.36[f]

[a] Viscosity measured with Brookfield Synchro-Lectric rotational viscometer, model HBT, 1 r. p. m., spindle 2.
[b] Viscosity measured with Brookfield Synchro-Lectric rotational viscometer, model HBT, 1 r. p. m., spindle 3.
[c] Viscosity measured with Brookfield Synchro-Lectric rotational viscometer, model HBT, 1 r. p. m., spindle 1.
[d] Viscosity measured with falling sphere viscometer (diameter of the sphere 0.3125 inch, weight 2.035 Gm.).
[e] Viscosity measured with Scott orifice efflux viscometer at atmospheric pressure.
[f] Viscosity measured with Scott orifice efflux viscometer at 50 mm. Hg pressure above atmospheric (increased rate of shear).
[g] The apparent viscosities of bases I and II overlap but at most of the rates of shear above 2.5 r. p. m. the apparent viscosities of base II are higher than that of base I (see Fig. 1).

Ung. Glycerin. Pharmacopoea Helvetica, 5th ed., as the base of 1% w/w acriflavine ointment is comparatively unstable; glycerin separates out, perceptible mold growth develops.

glass containers. The manner of impregnation of the gauze with the acriflavine emulsion ointment depends on whether a pourable ointment (base I, II, and IV) or a cream-like ointment (base III) is used. In case of the former, the ointment is directly poured to cover the gauze entirely, while with the latter type of ointment it should be premelted (at 44°) before pouring it. The containers are tightly closed and steam sterilized at 121° (15 lb./inch²) for 30 minutes. The applicability of the sterile acriflavine saturated gauze dressing is the same with whichever emulsion ointment it is prepared.

CONCLUSIONS AND SUMMARY

Thermosterilizable oil-in-water emulsion ointments of acriflavine are clinically preferred for topical antiseptic dressings. Emulsion ointments need not be oven-sterilized at 165° for 2 hours since the presence of water enables effective sterilization by autoclaving at 121°, 15 lb/inch², for 30 minutes only. The stability of thermolabile emulsion-ointments is substantially reduced by

TABLE II.—BACTERIAL INHIBITION ZONES[a] OF ACRIFLAVINE OINTMENTS

Bases	Acriflavine Content mM %, w/w	Gm. %, w/w	*Staph. aureus* 1/ud[b]	*Staph. aureus* 1/ud[b]	*E. coli* 1/ud[b]	*E. coli* 1/ud[b]	*Ps. aerug.* 1/ud[b]	*Ps. aerug.* 1/ud[b]	*Streptococcus pyogenes* 1/ud[b] A[c]	*Streptococcus pyogenes* 1/ud[b] B[c]	*Streptococcus pyogenes* 1/ud[b] A[c]	*Streptococcus pyogenes* 1/ud[b] B[d]
I	0	0	..	..	..	..	..	..	..	..	..	..
	0.35	0.1	2	2	..	..	..	..	4	4	5	5
	1.78	0.5	5	7	..	..	..	2	5	7	4	8
	3.55	1.0	6	8	..	2	1	3	4	7	4	8
	5.32	1.5	5	7	..	3	1	3	7	7	4	8
	7.1	2.0	5	7	..	3	1	4	6	8	5	8
II	0	0	..	..	..	..	..	..	..	..	..	..
	0.35	0.1	3	2	..	..	..	..	5	4	3	4
	1.78	0.5	4	4	..	..	..	..	3	4	7	4
	3.55	1.0	4	5	..	..	..	2	5	4	7	5
	5.32	1.5	4	5	1	1	4	4	7	7	6	6
	7.1	2.0	4	5	2	2	5	5	5	7	6	8
III	0	0	..	..	..	..	..	..	..	..	..	..
	0.35	0.1	3	3	..	..	..	..	2	3	5	10
	1.78	0.5	3	7	..	..	..	..	6	4	5	10
	3.55	1.0	3	7	..	2	2	..	6	5	7	10
	5.32	1.5	5	7	2	3	1	3	5	5	7	12
	7.1	2.0	5	7	2	4	1	4	7	6	7	12
IV	0	0	..	..	..	..	..	..	..	..	..	..
	0.35	0.1	2	3	..	..	..	..	4	4	4	3
	1.78	0.5	3	4	..	..	..	..	7	6	7	8
	3.55	1.0	4	6	1	..	..	1	6	6	6	8
	5.32	1.5	5	6	1	1	1	1	5	6	6	8
	7.1	2.0	5	5	1	1	2	2	5	6	6	8
Ung. Glycerin.[e]	0	0	..	..	..	..	..	..	..	..	..	..
	0.35	0.1	1	2	1	2	1	1	2	3	3	4
	1.78	0.5	3	4	1	3	2	1	4	5	5	6
	3.55	1.0	4	5	2	3	1	1	5	5	5	6
	5.32	1.5	4	5	2	1	1	1	5	6	6	6
	7.1	2.0	5	5	1	2	1	2	6	6	6	7

[a] Inhibition zones measured in millimeters, from one side of the ointment strip. [b] *d* = dilutions. [c] A = Simple Agar. [d] B = Blood Agar. [e] Ung. Glycerin. Pharmacopoea Helvetica: Amyl. Trit. 10 parts, Aq. dest. 15 parts, Glycerin 90 parts.

subjecting them to steam sterilization and the decomposition which ensues is irreversible.

Thermostable-sterilizable emulsions could be prepared by utilizing selected emulsifying agents properly blended. High temperature inversion point of an aqueous solution of an emulsifying agent *per se* signifies possible thermostability, while actual autoclave-thermotesting of the whole emulsion system containing the emulsifying agent determines conclusive thermostability. Both polysorbate 80 and polyoxyethylene 30 stearate exhibit inversion points at comparatively high temperatures. However, it was found that only polyoxyethylene 30 stearate mixed with auxilliary dispersion agents could produce thermosterilizable emulsion bases from which four bases of proper consistency were selected for the preparation of sterile acriflavine ointments.

The bacteriostatic activity of the sterile acriflavine emulsion ointments prepared with the selected bases is equivalent to that obtained with greaseless starch glycerite base with equal concentrations of acriflavine. It was confirmed that the least efficient concentration of acriflavine in water-miscible ointments is 1% which equals to 3.55 millimole %.

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A Study of Meprobamate-Curare Interaction*

By JAMES W. INGALLS, Jr.†, and LEO GREENBERG

Since both meprobamate and curare working through different sites and modes of action can produce the same gross effect of skeletal muscular relaxation, the possibility of synergistic interaction was investigated. The existence of such synergy has been demonstrated as an increased effectiveness and/or toxicity of curare in guinea pigs, rats, and mice pretreated with meprobamate.

ALTHOUGH meprobamate (Miltown, Equanil, 2-methyl-2-*n*-propyl-1,3-propanediol dicarbamate) was synthesized (1, 2) and originally investigated as a muscle relaxant (3–5), that aspect of its pharmacology was soon overshadowed in the public attention by its usefulness as a tranquilizer (6–8). Many clinical and laboratory investigators, however, have maintained interest in the muscle relaxant properties of this drug.

Clinical experience has shown beneficial responses in patients with cerebral palsy (9–11) and in patients with skeletal muscle spasm (12). Improvement has been seen in the general muscular coordination of patients and in their ability to perform tasks (13). In studies with laboratory animals, meprobamate has been used to control experimental epilepsy (14), and it has been shown that the muscular relaxation from meprobamate is potentiated by phenobarbital and extract of hyoscyamus (15, 16).

Investigation of the interaction between meprobamate and other drugs is of considerable import, not only because of the widespread use of the drug as a tranquilizer, but also because of the growing tendency to administer two or more drugs of the therapeutic armamentarium concomitantly. Such investigations have been underway at our laboratories since 1956 (17).

Study of interaction between meprobamate and curare seemed desirable from a practical viewpoint because curariform drugs are commonly employed in medical practice. They are established agents as adjuncts to general anesthesia as well as in convulsive shock procedures in psychiatric therapy. Furthermore, numerous European papers have recommended meprobamate as a preanesthetic medication in obstetrics (e. g., 18), and it is evident that occasional obstetrical patients become surgical patients, requiring general anesthesia. Meprobamate has also been suggested as a preoperative medication (19).

Pharmacologically, meprobamate and curare can produce the same gross effect of skeletal muscle relaxation but do so by completely different mechanisms (20, 21). Tubocurarine is a relatively short acting drug at the myoneural junction while meprobamate is a long acting drug in multineuronal circuits. Theoretically, synergy can be expected from two drugs which produce the same gross effect through different sites of action (22). It therefore seemed probable that there would be an interaction between meprobamate and those drugs which interrupt myoneural transmission.

MATERIALS AND METHODS

Animals were obtained from different sources and were usually adult males. They were fed commercial diets supplemented with fresh vegetables and kept in the thermostatically regulated animal house for several weeks prior to use. For each comparison conducted, the control and experimental animals used were from the same supplier, of the same sex, strain, and size range.

All drugs[1] were obtained in solution except for meprobamate which was suspended in 5% acacia solution, and were administered by intraperitoneal injection. The doses used were within the range of those employed by a great many other workers (e. g., 23, 24), the largest dose of meprobamate being 100 mg./Kg. Since loss of righting reflex is an important criterion in this study, it must be pointed out that in hundreds of experiments in our laboratories, this dose was completely ineffective in inducing a loss of righting reflex in guinea pigs, rats, and mice. Meprobamate was always injected thirty minutes prior to administration of the curariform agent after preliminary experiments showed that simultaneous administration of the slow-acting meprobamate with the rapidly-acting curariform drug was impractical.

Experiments were always conducted with paired groups of animals on the same day. Times were all taken to the nearest minute. The time for onset of paralysis was recorded when the righting reflex of the head was lost, and the time of recovery was noted at the reappearance of this reflex. The time of death was recorded at the cessation of heartbeat.

When there were qualitative differences in responses, the significance of difference between proportions was calculated. When qualitative re-

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[1] *d*-Tubocurarine chloride (Tubarine, Burroughs Wellcome), dimethyl-tubocurarine chloride (Mecostrin, Squibb), dimethyl-tubocurarine iodide (Metubine, Lilly), *Chondodendron tomentosum* extract (Intocostrin, Squibb), and meprobamate (Miltown, Wallace) were supplied through the courtesy of their manufacturers.

TABLE I.—MEPROBAMATE AND CURARE IN MALE GUINEA PIGS

Treatment, mg./Kg. Except Intocostrin, Units/Kg.	No. of Animals	Effect: No Paralysis	Effect: Paralysis and Recovery	Effect: Paralysis and Death	Type Synergy and Significance
2.0 Intocostrin	10	10	0	0	Increased effect
100 Miltown + 2 Intocostrin	10	5	4	1	$P < 0.02$
0.05 Mecostrin	10	10	0	0	Increased effect
100 Miltown + 0.05 Mecostrin	10	0	9	1	$P < 0.001$
0.1 Metubine	20	2	7	11	Increased toxicity
100 Miltown + 0.1 Metubine	20	1	1	18	$P < 0.05$
0.4 Tubarine	10	0	5	5	Increased toxicity
100 Miltown + 0.4 Tubarine	10	0	0	10	$P < 0.02$

The number of animals affected was significantly increased when meprobamate was combined with Intocostrin or Mecostrin and the number of deaths was significantly increased when meprobamate was combined with the other two drugs.

TABLE II.—MEPROBAMATE AND CURARE IN MALE MICE

Treatment mg./Kg. Except Intocostrin, Units/Kg.	No. of Animals	Effect: No Paralysis	Effect: Paralysis and Recovery	Effect: Paralysis and Death	Type Synergy and Significance
4.0 Intocostrin	10	7	0	3	Increased toxicity
100 Miltown + 4 Intocostrin	10	0	1	9	$P < 0.02$
0.4 Mecostrin	10	9	1	0	Increased toxicity
100 Miltown + 0.4 Mecostrin	10	0	3	7	$P < 0.01$
0.6 Metubine	10	5	1	4	Increased effect
100 Miltown + 0.6 Metubine	10	0	4	6	$P < 0.02$
0.4 Tubarine	10	10	0	0	Increased toxicity
100 Miltown + 0.4 Tubarine	10	4	0	6	$P < 0.01$

The number of animals affected was significantly increased when meprobamate was combined with Metubine, and the number of deaths was significantly increased when meprobamate was combined with the other three drugs.

TABLE III.—MEPROBAMATE AND CURARE IN MALE RATS

Treatment, mg./Kg. Except Intocostrin, Units/Kg.	No. of Animals	Effect: No Paralysis	Effect: Paralysis and Recovery	Effect: Paralysis and Death	Type Synergy and Significance
4.0 Intocostrin	7	0	0	7	Decreased onset
100 Miltown + 4 Intocostrin	7	0	0	7	time $P < 0.05$
0.1 Mecostrin	10	0	0	10	Decreased survival
100 Miltown + 0.1 Mecostrin	10	0	0	10	time $P < 0.001$
0.01 Metubine	10	10	0	0	Increased effect
100 Miltown + 0.01 Metubine	10	5	4	1	$P < 0.02$
0.2 Tubarine	11	0	8	3	Increased toxicity
100 Miltown + 0.2 Tubarine	11	0	3	8	$P < 0.05$

The number of animals affected was significantly increased when meprobamate was combined with Metubine, and the number of deaths was significantly increased when meprobamate was combined with Tubarine. The time of onset of action of 5.1 ± 0.5 minutes for Intocostrin was significantly reduced to 3.4 ± 0.4 minutes by pretreatment with meprobamate. The length of time of survival of 10.4 ± 0.4 minutes for Mecostrin was reduced to 6.1 ± 0.7 minutes by pretreatment with meprobamate.

TABLE IV.—ACUTE TOXICITY TUBARINE ALONE AND FOLLOWING PRETREATMENT WITH MILTOWN (GROUPS 10 GUINEA PIGS)

Tubarine: mg./Kg.	Tubarine: Mortality	Tubarine: LD_{50}, mg./Kg.	100 mg./Kg. Miltown + Tubarine: mg./Kg.	100 mg./Kg. Miltown + Tubarine: Mortality	100 mg./Kg. Miltown + Tubarine: LD_{50}, mg./Kg.
0.30	0	..	0.10	1	..
0.35	2	..	0.20	4	..
0.40	5	0.42	0.30	6	0.24
0.45	6	..	0.40	10	..
0.50	7	..			

The difference between the means of the doses lethal for animals pretreated with meprobamate and those not so pretreated is very highly significant with $P < 0.001$. This may be interpreted to mean that pretreatment with 100 mg./Kg. meprobamate thirty minutes before administration of Tubarine was equivalent to a 75% increase in the dose of Tubarine.

sponses were the same for all animals of both groups (e. g., all were paralyzed and recovered, or all died), t tests were applied to demonstrate the significance of quantitative differences. The times for onset of

TABLE V.—CROSSOVER TREATMENT IN FEMALE RATS

Group	Treatment, mg./Kg.	No. of Animals	Effect: Paralysis and Recovery	Effect: Paralysis and Death
A	0.2 Tubarine	8	7	1
B	100 Miltown + 0.2 Tubarine	8	3	5
A Survivors	100 Miltown + 0.2 Tubarine	7	0	7
B Survivors	0.2 Tubarine	3	3	0

Following the initial treatments the difference in percentage fatalities was not significant, but there was a significant difference in times of onset of paralysis for the two groups, with $P < 0.02$. When the crossover was done three days later, all animals which had survived tubocurarine alone were killed by the combination, and no animal which had survived the combination was killed by the tubocurarine alone, $P < 0.01$.

TABLE VI.—EFFECTS OF SMALL DOSES ON GUINEA PIGS AND RATS

Treatment, mg./Kg.	Species	No. of Animals	Effect		
			No Paralysis	Paralysis and Recovery	Paralysis and Death
25 Miltown + 0.2 Tubarine	G. P.	7	7	0	0
50 Miltown + 0.2 Tubarine	G. P.	7	6	1	0
75 Miltown + 0.2 Tubarine	G. P.	7	3	4	0
0.1 Tubarine	Rat	8	8	0	0
50 Miltown + 0.1 Tubarine	Rat	8	6	2	0
100 Miltown + 0.1 Tubarine	Rat	8	5	2	1
0.2 Tubarine	Rat	8	3	3	2
50 Miltown + 0.2 Tubarine	Rat	8	0	4	4

With guinea pigs, results from pretreatment with 50 mg./Kg. were not significantly greater than with 25 mg./Kg., nor were results with 75 mg./Kg. significantly greater than with 50 mg./Kg., but pretreatment with 75 mg./Kg. was significantly more effective than with 25 mg./Kg., $P < 0.05$. Although these combinations were apparently nontoxic, none was consistently effective, and since 100 mg./Kg. meprobamate plus 0.2 mg./Kg., Tubarine had previously killed 40% of the test animals, it did not appear worthwhile to pursue the search for a combination of dosages that would be both consistently effective and uniformly safe in guinea pigs. With rats, there was likewise no substantial margin of safety; no combination which could be relied upon to produce paralysis without producing deaths.

paralysis, duration of paralysis, and survival are recorded as the mean time plus or minus the standard error.

RESULTS AND DISCUSSION

Synergy between meprobamate and curare in guinea pigs, mice, and rats can be seen from the results given in Tables I, II, and III.

In addition to the synergistic effects previously recorded, a change in the LD_{50} can be demonstrated by expanding the range of doses, as illustrated by Table IV.

A comparison of the results in the previous table shows that a nontoxic dose of meprobamate (100 mg./Kg.) followed by a nontoxic dose of tubocurarine (0.1, 0.2, 0.3 mg./Kg.) formed a highly dangerous combination for these guinea pigs. When crossover tests were conducted to compare the actions of curare alone and curare after meprobamate in the same animals, the results were always the same; the combination of drugs was more effective, i. e., more toxic. This type of response is illustrated in Table V.

In the hope that a reliably effective, nontoxic combination of doses might be obtained, smaller doses of meprobamate were used as pretreatments, and pretreatments were given before smaller doses of curare. This type of experiment is illustrated by Table VI.

It was, however, possible to observe a subparalytic interaction of the drugs. That is, a dose of tubocurarine which had no subjectively determinable effect on rats could be seen to produce muscular relaxation in rats which had been pretreated with a small dose of meprobamate. When rats were given 0.15 mg./Kg. tubocurarine, there was no obvious gross effect on the musculature or activity. At the same time, rats pretreated with 20 mg./Kg. meprobamate were given 0.15 mg./Kg. tubocurarine and although none of this group became paralyzed, their musculature and activity were both affected. These latter animals flattened down in the bottom of the cage and could be pushed about readily by hand, although they resisted being placed on their sides. The amount of meprobamate used in this experiment does not in itself have a grossly demonstrable effect on the skeletal muscle of the rat.

Apparently this synergistic type of action with meprobamate is not limited to the curares, but extends to the curarimimetic drugs. Thus, we have seen significant interaction with succinylcholine chloride and with gallamine trimethiodide, and presume that it is a general effect.

SUMMARY

The studies described have demonstrated the synergy between meprobamate and curare. The toxicity from a given dose of curare was increased in animals which had been pretreated half an hour before with a nontoxic, nonparalytic dose of meprobamate.

During these studies, no combination of dosages of these drugs was encountered which would paralyze all animals without killing any.

It was possible, however, to observe a nonparalytic decrease in skeletal muscle tone of the rat after a combination of a low, ineffectual dose of meprobamate followed by a low, ineffectual dose of tubocurarine.

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Isotonic Solutions VIII*

The Permeability of Red Corpuscles to Various Salts of Gluconic Acid

By HOWARD C. ANSEL† and WILLIAM J. HUSA

A comparison was made of van't Hoff *i* values calculated from hemolytic and freezing point data for various salts of gluconic acid. Sodium, potassium, and manganese(II) gluconates gave higher *i* values by the hemolytic method than by the freezing point depression method; iron(II) and cobalt(II) gluconates gave lower *i* values by the hemolytic method. Magnesium and calcium gluconates gave higher *i* values by the hemolytic method with human erythrocytes but not with rabbit erythrocytes. Zinc gluconate gave extremely high hemolytic *i* values due to the partial precipitation of the oxyhemoglobin liberated from laked erythrocytes. Hemolytic *i* values of the gluconates were generally lowered when determined in the presence of sodium chloride. Erythrocytes from Negro donors were, on the average, more resistant to osmotic hemolysis than those from Caucasian donors. Results substantiate the premise that solutions calculated to be isosmotic with blood according to colligative property data are not necessarily isotonic to the red corpuscle.

THE ADJUSTMENT of solutions intended for intravenous administration to the same tonicity as blood is of utmost importance. The advantages of employing the hemolytic method in the preparation of such solutions have been emphasized by Husa and co-workers (1–7). They pointed out that the osmotic effect of a substance on the erythrocyte depends not only on the concentration of the substance but also on whether or not it penetrates or affects the properties of the cell membrane. Thus, solutions calculated to be isosmotic with blood according to physicochemical data are not necessarily isotonic to the red blood cell.

Cadwallader and Husa (8) recently reported that zinc acetate gave unusually high hemolytic *i* values, much beyond that calculated from colligative property data. Their work complemented that of Hartman and Husa (4), who found similarly high hemolytic *i* values for zinc sulfate.

The primary purpose of the present work was to investigate the elements closely related to zinc in the periodic table to determine whether or not their action paralleled that of zinc. Added interest in the investigation was stimulated by the fact that these elements, i. e., copper, cobalt, iron, and manganese, along with zinc are so-called "trace elements," essential to human nutrition. To obtain a comparative picture of the activities of these elements, they were all employed as salts of gluconic acid. Sodium, potassium, magnesium, and calcium gluconates were also studied and their hemolytic *i* values determined. The *i* values obtained by the hemolytic method were compared to *i* values calculated from freezing point depression data collected in this laboratory.

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This paper is based in part upon a dissertation presented to the Graduate Council of the University of Florida by Howard C. Ansel, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The gluconates used in this study were generously supplied by Chas. Pfizer & Co., Inc., Brooklyn 6, N. Y., and Chemo Puro Manufacturing Corp., Newark 5, N. J.

EXPERIMENTAL

Collection of Blood.—The rabbit and human bloods employed in this investigation were obtained in the manner described by Grosicki and Husa (2). The human blood was drawn chiefly from the veins of the arms of a twenty-five-year-old male, although some experiments were conducted employing blood donated by male volunteers.

Preparation of Solutions.—Solutions of the chemicals were prepared by placing the proper amounts in volumetric flasks which were then brought to volume with triple-distilled water. An approximate concentration range was determined for each gluconate by conducting a preliminary experiment in which the concentration of gluconate just sufficient to prevent hemolysis and the maximum concentration present without the prevention of hemolysis constituted the limits of the desired range. Various intermediate concentrations were then prepared and the experiment carried out. All of the chemical concentrations were calculated in terms of the anhydrous salt.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis was that of Hunter (9), as adapted for the determination of *i* values of salts forming colorless solutions by Grosicki and Husa (2). Several gluconates studied in the present investigation formed colored aqueous solutions, e.g., cobalt(II), copper(II), iron(II), and manganese(II) gluconates. For the quantitative determination of the per cent hemolysis when studying such solutions, one of two methods was employed depending on whether or not the color of the gluconate solution produced an additive colorimetric reading with oxyhemoglobin solution.

In the first method, oxyhemoglobin solution pre-

pared by laking erythrocytes in triple-distilled water was added in like amounts to each of two colorimeter tubes, one containing 5 ml. of triple distilled water and the other containing 5 ml. of the gluconate solution. To a third tube, containing 5 ml. of the gluconate solution, a portion of triple distilled water equal in volume to the oxyhemoglobin solution placed in each of the first two tubes was added. The colorimetric reading of the third tube added to the reading of the tube containing only water and oxyhemoglobin was compared to the reading of the tube containing the oxyhemoglobin and the colored gluconate solution; if the readings agreed within experimental error, the two colors were considered to be additive. If this were found to be the case, colorimeter readings of the gluconate solutions were taken before the addition of blood and served as blank readings. These blank readings subtracted from the colorimetric readings obtained at the end of the hemolytic experiment permitted an accurate determination of the proportion of light absorbed by the oxyhemoglobin present in the supernatant liquid.

If the colorimetric readings of the oxyhemoglobin and the gluconate solutions were not found to be additive, the second of the two methods devised was employed. In this method the per cent hemolysis was determined by difference, that is, from the proportion of oxyhemoglobin remaining in the unlaked erythrocytes. The standard procedure used when employing colorless solutions was carried out until just prior to the time that the colorimetric readings were to be taken. Instead of taking the readings, the supernatant solution, containing the colored gluconate and oxyhemoglobin from laked cells, was meticulously decanted from each tube, being careful not to disturb the unhemolyzed cells spun to the bottom of the tubes. The unhemolyzed cells were then washed with 0.85% sodium chloride solution, recentrifuged, and the supernatant liquid decanted, the process being repeated until the washings were free of color. The supernatant liquid of the last washing was replaced with 0.1% sodium carbonate solution added to a mark etched on the colorimeter tube, denoting a volume of 5.05 ml. The tube was inverted several times to insure complete hemolysis, the ghost cells centrifuged down, and the resulting color read with the colorimeter. By subtracting the average reading of two duplicate tubes from the average reading of 100% hemolysis, the colorimetric value of the oxyhemoglobin exuded from the cells originally hemolyzed in the gluconate solution was obtained. From this value the per cent of hemolysis was calculated in the usual manner (2). All of the hemolysis experiments were conducted at 37° ± 1°.

Calculation of i Values from Per Cent Hemolysis Data.—The calculation of hemolytic i values has been explained by Husa and co-workers (2, 3).

Determination of Metal-Protein Precipitation.—To aid in the interpretation of certain hemolytic i values, tests were carried out to determine whether or not precipitation occurred when solutions of oxyhemoglobin were added to solutions of the gluconates. One % solutions of each gluconate were separately mixed with equal portions of oxyhemoglobin solution prepared by laking washed erythrocytes, allowed to stand in a water bath at 37° for forty-five minutes, centrifuged, and observed for evidence of precipitation. During the course of a hemolytic run, such precipitation would cause the colorimetric readings to be proportionally lower than those which would normally result from the proportion of oxyhemoglobin actually released from the laked erythrocytes.

Additional experiments were carried out to determine whether or not serum proteins were precipitated by gluconate solutions. Serum was separated from blood cells by centrifugation and added in like portions to 1% solutions of each gluconate. The mixtures were observed repeatedly for forty-five minutes during which time they were in a water bath at 37°.

Freezing Point Determinations.—The apparatus used to determine the freezing points of 0.010 molal (for calcium gluconate) and 0.025 molal gluconate solutions was that of Bartley (10), as modified by Husa and Adams (1). The average freezing point depression of water for ten samples of each gluconate solution was determined and the van't Hoff i values calculated according to the following formula

$$\Delta T_f = K_f i M$$

where ΔT_f is the change in the freezing point of water after the addition of solute, K_f is the molal freezing point constant of water (1.86), i is the van't Hoff factor, and M is the molality of the solution.

RESULTS AND DISCUSSION

Results With Sodium and Potassium Gluconates.—Employing the hemolytic method, sodium and potassium gluconates gave higher i values than would be expected for compounds yielding two ions. Freezing point information indicated that each of these gluconates acted normally, i.e., as slightly less than two particles, see Table III.

Sodium gluconate exhibited an average i value of 2.27 using rabbit blood and 2.37 using human blood. These values were lowered to 1.99 with rabbit blood and 2.07 with human blood when determined in the presence of 0.2% sodium chloride.

Potassium gluconate gave an average i value of 2.26 using rabbit blood and 2.28 using human blood. These values were lowered to 2.05 with rabbit blood and 2.13 with human blood when the determinations were conducted in the presence of 0.2% sodium chloride, see Tables I and II.

In solutions of some substances, erythrocytes may lose electrolyte from within the cell, thus causing a decrease in the internal osmotic pressure and a corresponding increase in resistance to hemolysis (2, 11, 12). The exosmosis of erythrocytes in such solutions can be corrected in some cases by the addition of small proportions of an electrolyte, e. g., sodium chloride (2, 12).

The high hemolytic i values obtained for sodium and potassium gluconates may be attributed to exosmosis of electrolyte from within the erythrocytes. The lowering of the i values upon the addition of sodium chloride may be due to the partial restoration of the environment normal to the red blood cells.

Results With Calcium and Magnesium Gluconates.—The hemolytic i values for calcium gluconate, calculated from concentrations causing 25, 50, and 75% hemolysis of human erythrocytes, increased by approximately 0.2 (2.54 to 2.72) going from 25 to 50% hemolysis and another 0.2 (2.72

TABLE I.—VALUES OF *i* FOR VARIOUS GLUCONATES CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT ERYTHROCYTES[a]

Gluconate	Hemolysis, % 25	50	75	Averages
Sodium	2.18	2.26	2.36	2.27
Sodium[b]	1.96	1.97	2.05	1.99
Potassium	2.19	2.24	2.34	2.26
Potassium[b]	2.00	2.07	2.08	2.05
Magnesium	2.48	2.64	2.76	2.63
Magnesium[b]	3.03	3.19	3.30	3.17
Calcium	2.32	2.40	2.50	2.41
Calcium[b, c]	2.89	2.96	3.12	2.99
Manganese (II)	2.48	2.54	2.62	2.55
Manganese (II)[b]	2.04	2.18	2.36	2.19
Iron(II)	1.22	1.28	1.32	1.27
Iron(II)[b]	0.93	1.11	1.69	1.24
Cobalt(II)	2.21	2.27	2.48	2.32
Zinc	793.00	933.00	1027.00	918.00

[a] Unless otherwise indicated all *i* values represent an average of two blood samples.
[b] Values determined in the presence of 0.2% sodium chloride.
[c] Average of three blood samples.

TABLE II.—VALUES OF *i* FOR VARIOUS GLUCONATES, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

Gluconate	Hemolysis, % 25	50	75	Averages
Sodium	2.32	2.36	2.42	2.37
Sodium[b]	2.01	2.07	2.14	2.07
Potassium	2.20	2.28	2.36	2.28
Potassium[b]	2.08	2.13	2.18	2.13
Magnesium[c]	3.36	3.94	4.69	4.00
Magnesium[b]	3.34	3.40	3.56	3.43
Calcium	2.54	2.72	2.95	2.74
Calcium[b]	2.41	2.56	2.89	2.62
Manganese(II)	2.64	2.70	2.76	2.70
Manganese(II)[b]	2.28	2.37	2.47	2.37
Iron(II)	1.45	1.58	1.79	1.61
Iron(II)[b]	0.74	1.12	2.56	1.47
Cobalt(II)	1.98	2.08	2.18	2.08
Zinc	...[d]	499.00	666.00	582.00

[a] Unless otherwise indicated all *i* values represent an average of two blood samples.
[b] Values determined in the presence of 0.2% sodium chloride.
[c] Average of three blood samples.
[d] Data unobtainable at 25% hemolysis.

to 2.95) going from 50 to 75% hemolysis. A similar observation was made by previous investigators of calcium gluconate, acetate, and levulinate (5). This steady increase did not occur when the *i* values for calcium gluconate were determined in the presence of 0.2% sodium chloride, see Table II.

Hemolytic *i* values for calcium gluconate obtained with rabbit blood increased by approximately 0.1 (2.32 to 2.40) going from 25 to 50% hemolysis and by another 0.1 (2.40 to 2.50) going from 50 to 75% hemolysis. Increases of similar magnitude occurred when the determinations were conducted in the presence of 0.2% sodium chloride.

The presence of 0.2% sodium chloride caused an increase in the *i* values for calcium gluconate using rabbit blood and a decrease in the *i* values when human blood was employed. In this respect, magnesium gluconate acted similarly to calcium gluconate.

TABLE III.—VALUES OF *i* FOR VARIOUS GLUCONATES, CALCULATED FROM FREEZING POINT DATA OBTAINED WITH A MODIFIED BARTLEY APPARATUS[a]

Gluconate	Average Freezing Point Depression, °C.	*i* Value
Sodium	0.087	1.87
Potassium	0.085	1.83
Magnesium	0.130	2.80
Calcium[b]	0.050	2.69
Manganese(II)	0.102	2.19
Iron(II)	0.114	2.45
Cobalt(II)	0.112	2.41
Copper(II)	0.069	1.48
Zinc	0.119	2.57

[a] Unless otherwise indicated data were obtained from 0.025 molal gluconate solutions.
[b] Data obtained from 0.010 molal solutions.

The *i* values obtained for magnesium gluconate were higher in every analogous case than those obtained for calcium gluconate. Employing human blood, an average *i* value of 4.00 was obtained for magnesium gluconate which was reduced to 3.43 when determined in the presence of 0.2% sodium chloride. These *i* values are much beyond that calculated for magnesium gluconate from freezing point data, *viz.* 2.80. Using rabbit blood, the *i* values for magnesium gluconate increased from 2.63 to 3.17 when the determinations were carried out in the presence of 0.2% sodium chloride.

Under normal conditions, calcium does not penetrate the red blood cell (13). According to Ponder (14) there is considerable exchange of internal potassium for alkaline earth metals in human cells under certain conditions. Davson and Danielli (15) noted the difference in the response of the cat erythrocyte membrane to sodium and potassium permeability in the presence of the alkaline earths; they showed that tonicity changes were responsible for only a portion of the abnormal reactions of the membrane. The solidifying influence of calcium has often been noted (16); the alkaline earths were said to thicken the erythrocyte membrane by forming insoluble salts with the phosphatide acids of the cell wall (17). Danielli (18) mentioned that there is a good deal of evidence that substances such as magnesium act primarily on cell surfaces, in some way modifying the excitability of the cells.

It is not unlikely that the results obtained for magnesium and calcium gluconates were largely due to the action of the cations on the cell membrane, in some manner altering the normal permeability characteristics of the cell. The presence of 0.2% sodium chloride along with the alkaline earth gluconates seemed to promote exosmosis of rabbit erythrocytes and to prevent it with human erythrocytes.

Results With Cobalt(II) and Manganese(II) Gluconates.—Cobalt(II) gluconate gave *i* values of 2.32 using rabbit erythrocytes and 2.08 with human erythrocytes; freezing point data indicated an *i* value of 2.41. The lower *i* values obtained by the hemolytic method may be attributed, in part, to the acidity of the cobalt(II) gluconate solutions (about pH 5); such acidity could cause hemolysis independent of the osmotic pressure of the solution. In solutions containing 0.6% sodium chloride and 2% cobalt(II) gluconate, there was approximately 10% hemolysis of human erythrocytes and essentially no

hemolysis of rabbit erythrocytes; the hemolysis of the human erythrocytes would be necessarily due to something other than osmotic effects and could account for the lower hemolytic i values obtained with human blood than with rabbit blood.

Manganese(II) gluconate gave i values of 2.55 with rabbit erythrocytes and 2.70 with human erythrocytes. In the presence of 0.2% sodium chloride, the i values obtained using each blood type were lowered by approximately the same amount, i. e., 0.33 with human blood and 0.36 using rabbit blood. The lower i values more closely resembled the i value obtained for manganese(II) gluconate by the freezing point depression method, *vis.*, 2.19.

Results With Iron(II) Gluconate.—Employing the hemolytic method, iron(II) gluconate gave i values of 1.27 with rabbit blood and 1.61 with human blood. These i values were lowered to 1.24 using rabbit blood and 1.47 with human blood when the determinations were carried out in the presence of 0.2% sodium chloride. These i values are all lower than would be expected for a compound yielding three ions; an i value of 2.45 was calculated for iron(II) gluconate from freezing point data.

Throughout the course of this investigation, there was a tendency to be doubtful of the stability of the iron(II) gluconate solutions. Stone (19) stated that although iron(II) gluconate is stable in the solid state, its solutions are rather unstable. They are prone to oxidation, with iron(II) changing to iron (III), the rate of transformation being dependent on the pH, temperature, degree of aeration, presence of oxidizing or reducing agents, etc. Oxidation causes a darkening of the solutions, a characteristic which would make certain colorimetric assays unreliable. An iron(III) compound of unknown composition has been found to precipitate from aqueous solutions of iron(II) gluconate as a result of a photochemical reaction.

Although iron(II) gluconate solutions were prepared just prior to use, precipitates were occasionally noticed in the colorimeter tubes during some experiments. These brown colored precipitates generally occurred in the iron(II) gluconate-blood mixtures containing the lower proportions of the iron(II) salt. If a portion of the iron(II) gluconate were precipitated as an oxidized form, fewer particles would be in solution to aid in the prevention of osmotic hemolysis; the resulting data would indicate less protection and thus a low i value.

The binding of iron(II) gluconate by plasma proteins (e. g., beta-globulins), with or without precipitation, would decrease the number of particles in solution and the resulting i values would be lower than expected. The pH of the iron(II) gluconate solutions was approximately 4.5, remaining about the same during the course of each determination; this acidity could account, to a small degree, for the low i values. In the presence of 0.6% sodium chloride, iron(II) gluconate seemed to cause appreciable hemolysis of human erythrocytes but not of rabbit erythrocytes; in the experiments employing human blood, however, precipitation was observed.

Results With Copper(II) Gluconate.—Copper(II) gluconate in concentrations of from 1 to 10% seemed to cause denaturation of blood; oxyhemoglobin released from laked erythrocytes turned brown as did the unhemolyzed red blood cells. The pH of the copper(II) gluconate solutions was approximately 4.0, this pH remaining fairly constant for the duration of the experiments. Bloch and Oelsner (20) reported that at pH 3.0 hemoglobin is instantaneously converted to brown hematin, at pH 3.8 it is converted within twenty-five minutes, and at pH 4.6 the formation of hematin is incomplete within twenty-four hours. They stated that the rate of conversion is directly proportional to the hydrogen ion concentration.

In the tests conducted to determine the protein precipitating action of copper(II) gluconate, it was found that both rabbit and human blood serum protein were precipitated to a certain extent by this gluconate. The copper(II) gluconate-serum protein precipitate redissolved on standing (and instantaneously on agitation) and was milk-white in color. The addition of alkali to the copper(II) gluconate-serum mixture resulted in a voluminous precipitate. Ressler, *et al.* (21), found that copper combined in definite proportions with the albumin and gamma-globulin of blood serum.

Although hemolytic i values were unobtainable due to the darkening of the blood, freezing point data gave an i value of 1.48 for copper(II) gluconate which was appreciably lower than the usual value of 2.70 for a compound yielding three ions.

Results With Zinc Gluconate.—Employing the hemolytic method, extremely high i values were obtained for zinc gluconate The values of i at concentrations causing 25, 50, and 75% hemolysis of rabbit erythrocytes were 793, 933, and 1,027, respectively, whereas concentrations causing 50 and 75% hemolysis of human erythrocytes showed i values of 499 and 666, respectively. Values of i at 25% hemolysis of human erythrocytes were unobtainable.

Throughout the course of this investigation, unusual behavior was noticed between blood and zinc gluconate solutions. Blood appeared to agglutinate as it was added to colorimeter tubes containing solutions of the zinc salt; at the same time, a milk-white formation presented itself around the blood. Before inverting the tubes (to mix their contents) a layer of clear oxyhemoglobin solution was observed at the bottom of some of the tubes; after inverting the tubes, the contents became uniformly opaque. A sediment was observed at the bottom of each tube after centrifugation which was of a volume greater than could be attributed to the small proportion of blood added. The substance was of a reddish color (resembling cosmetic rouge) and was amorphous under the microscope. The degree of hemolysis was determined in the usual manner and the i values calculated according to the general methods.

Oxyhemoglobin solution was partially precipitated when placed in contact with a 1% solution of zinc gluconate. The precipitate was soluble below pH 5.5 and above pH 10.0; it appeared to be least soluble at about the neutral point. Oxyhemoglobin solutions prepared from human erythrocytes acted in a similar manner. Human and rabbit serums were each precipitated in the presence of zinc gluconate. The white, curdy precipitates were soluble below pH 5.0. Oxyhemoglobin was more effectively precipitated by zinc gluconate when a proportion of serum was present. Also, when serum and oxyhemoglobin were separately added to a zinc gluco-

nate solution, the precipitation was less complete than when the two proteins were mixed before being added. Rawlinson (22) described a zinc-oxyhemoglobin compound precipitated from a solution of rabbit blood pigment by zinc sulfate. Gurd (23) said that both gamma-globulin and beta-lipoprotein can be precipitated in the presence of zinc acetate, with the former aiding in the precipitation of the latter.

When blood was added to a solution containing 1% of zinc gluconate and 0.85% of sodium chloride, the blood cells settled to the bottom of the tube and failed to hemolyze after forty-five minutes at 37°. After centrifugation, the cells were bound together at the bottom of the tube and failed to hemolyze after the supernatant solution was decanted and replaced with triply distilled water. Inversion of the tubes caused the cells to pass through the water, still bound together, and resembling a "pancake." Gibson (13) reported that red cells take up zinc very readily; whether the zinc enters the cells or is bound to the membrane is not known. It has been shown, however, that the zinc cannot be removed by washing in isotonic sodium chloride solution.

It appears obvious that the high hemolytic i values obtained for zinc gluconate in this work and for zinc sulfate and acetate in other works (4, 8) have been due to the precipitation of the oxyhemoglobin liberated from laked erythrocytes which made the colorimetric readings of the degree of hemolysis misleading.

Variability of Blood.—The osmotic fragility of erythrocytes varied from animal to animal and, indeed, from day to day in the same animal; employing blood from the same donor, diurnal variations were observed in the degree of hemolysis occurring in a given concentration of sodium chloride solution.

Human blood generally produced greater colorimetric readings for 100% hemolysis than did rabbit blood. Negro blood employed in two experiments early in this study failed to become 60% hemolyzed in 0.32% sodium chloride solutions, a salt concentration which in most instances seemed to completely lake erythrocytes from White donors. Eight additional blood samples from Negro volunteers were obtained and the osmotic fragility of the erythrocytes determined in various concentrations of sodium chloride. Although two of the ten Negro blood samples exhibited greater than 90% hemolysis in 0.32% sodium chloride solutions, seven samples showed less than 80% and three samples less than 60% hemolysis. An average of 74.7% hemolysis was shown by the ten Negro blood samples in 0.32% sodium chloride; the samples of Caucasian donors' blood (30 blood samples) employed in this study showed an average of 97.0% hemolysis in 0.32% sodium chloride solutions. Blood from Caucasians showed, on the average, 50% hemolysis in sodium chloride solutions ot 0.381% concentration; the same degree of hemolysis did not occur in blood from Negroes until the sodium chloride concentration was decreased, on the average, to 0.342%. All of the Caucasian blood samples hemolyzed to a certain degree in 0.42% sodium chloride concentration whereas only four of the Negro blood samples exhibited hemolysis in this salt concentration.

Diseases of the blood and other pathological conditions have long been known to cause a variation in the osmotic fragility of erythrocytes (24). Sickle cell anemia, a disease occurring almost entirely in the Negro race, is characterized by sickle-shaped erythrocytes and by an increase in the resistance of these cells toward hypotonic saline. In the present study, there was no evidence of sickling observed in the Negro blood samples.

SUMMARY

1. Hemolytic i values for various salts of gluconic acid have been determined and compared with i values calculated from freezing point depression data.

2. A procedure for the determination of the degree of hemolysis of erythrocytes suspended in colored aqueous solutions has been devised.

3. Using the hemolytic method, sodium and potassium gluconates gave i values which were approximately 25 per cent higher than those obtained by the freezing point depression method; this difference was only half as great when the hemolytic determinations were conducted in the presence of 0.2 per cent sodium chloride.

4. Magnesium and calcium gluconates gave higher i values by the hemolytic method than by the freezing point depression method when human erythrocytes were employed and lower ones when rabbit erythrocytes were used. The addition of 0.2 per cent sodium chloride raised the hemolytic i values of these salts when rabbit blood was used and lowered the values when human blood was used.

5. Manganese (II) gluconate gave slightly higher and cobalt (II) gluconate slightly lower i values from hemolytic data than from freezing point data.

6. The unusually low hemolytic i values obtained for iron (II) gluconate were, in the main, attributed to the instability of the solutions.

7. Zinc gluconate gave extremely high hemolytic i values due to the partial precipitation of the oxyhemoglobin liberated from laked erythrocytes making the colorimetric readings of the supernatant liquid misleading.

8. Erythrocytes from Negro donors were found to be, on the average, more resistant to osmotic hemolysis than those from Caucasian donors.

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Studies on the Adsorption of Odorous Materials II*

Surface Potential Changes Due to the Adsorption of Alcohol Vapors

By J. O. KOPPLIN†, J. R. EATON, and J. E. CHRISTIAN

The normal homologous alcohol vapors from 2- to 8-carbon-chain lengths were injected into an air stream in concentrations varying from 0.2 to 500 parts of alcohol vapor per million parts of air by volume and were passed over a water surface. Relationships between changes in the surface potential of the water surface and the chain length of the adsorbed alcohol molecules were determined and they were found to be analogous to the relationships between the olfactory intensities of the same alcohol vapors and the chain length of the vapors as reported by other research groups. The results of these tests suggest that the adsorption and surface potential change processes are influenced by the structure of the adsorbate molecule—a factor generally associated with odor.

IN A PREVIOUS PAPER (1), changes in surface potential caused by the adsorption of various vapors on solid or liquid adsorbing surfaces were discussed. The various vapors in very low concentrations by volume were injected into an air stream which passed over the adsorbing surface. The effects of changes in the humidity of the air stream and changes in the temperature of the adsorbing surface were presented along with a report on the application of radioactive tracer techniques for the measurement of the amount of vapor adsorbed.

In this paper the changes in the surface potential caused by the adsorption of several normal homologous alcohol vapors of a distilled-water surface are presented. These changes were determined by measuring the contact potential difference between two surfaces, using as a reference one surface the potential of which did not vary. The Kelvin method for measuring contact potential differences as modified by Zisman (2) was used in this investigation. A description of the apparatus and general test procedure used is given in the preceding paper (1).

Because of the interest in the possible development of an objective instrument for the measurement of odor and because current opinion is that physical rather than chemical processes are critical in olfactory stimulation, the normal aliphatic homologous alcohols were studied as odorous air stream contaminants. Besides the fact that these alcohols can be smelled by the human nose, they are rather simple, well known organic substances concerning which considerable physical data are available—such as molecular weight, vapor pressure, dipole moment, and solubility.

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EXPERIMENTATION AND DISCUSSION

Ethyl alcohol was adopted as a standard test vapor to check and to calibrate the system whenever a test was run or whenever the water surface was replaced. Except in a very few cases not more than three injections of any alcohol vapor were made into the air stream before the water surface was replaced. This was done because of the general loss in sensitivity of the surface with time and with repeated injections of contaminant vapor into the air stream. Surface sensitivity for a particular alcohol vapor in the air stream, as used in this paper, is measured by the ratio of the change in surface potential expressed in millivolts (mv.) to the alcohol vapor concentration in the air stream expressed in parts per million by volume (p. p. m.).

For comparing the different alcohol vapors, measurement of the amount of vapor injected into the air stream was made in two different ways. The most often used measurement was in terms of parts per

million by volume of alcohol vapor in the air stream. This was calculated using the product of two ratios—the ratio of the volume of saturated vapor injected into the air stream per unit of time to the volume of air passing over the surface per unit of time, and the ratio of the vapor pressure in mm. of mercury of the particular alcohol used to standard atmospheric pressure in mm. of mercury. Thus, the injection of two different alcohol vapors into the air stream at the same concentration in p. p. m. by volume required a greater volume of saturated vapor of the alcohol having the lower vapor pressure. The second method was the injection of the various saturated vapors at equal rates (ml. per second) into the air stream. This resulted in a smaller number of alcohol molecules in the air stream for each lower vapor pressure alcohol.

Ethyl Alcohol Vapor.—After approximately forty separate tests were run using different rates of injection of ethyl alcohol vapor into the air stream with different distilled water surfaces, the accumulated data were carefully analyzed. It was found from considering all of the first injections of ethyl alcohol vapor on each surface that the sensitivity of the individual water surfaces varied, but many water surfaces indicated good agreement in the mv. change in surface potential as caused by a certain number of p. p. m. by volume of ethyl alcohol vapor in the air stream. The change in surface potential was taken as the difference between the surface potential at the time injection of contaminant vapor into the air stream was started and the final equilibrium value of the surface potential with the contaminant laden air stream. If the surface potential were slowly drifting at the time the test was made, a correction was made to cancel the effect of the drift. Picking out various sets of data which were in close agreement as to the change in potential for a given vapor concentration, it was found that, if the mv. change in the surface potential were plotted against the logarithm of the alcohol vapor concentration in the air stream in p. p. m. by volume, a straight line relationship was indicated. This suggested the possibility of an exponential relationship between the p. p. m. of ethyl alcohol vapor in the air stream and the mv. change of the surface potential within the range tested. For ethyl alcohol the variation in p. p. m. was from 30 to 500.

As a check on this exponential relationship, a single distilled-water surface was used and ethyl alcohol vapor was injected six times—each succeeding time at an increased concentration in the air stream. As a check on the surface sensitivity, a seventh injection of ethyl alcohol vapor was made at the same concentration as the first injection. The difference in the change in surface potential caused by the first and last vapor injections was taken as a measure of the total loss in sensitivity of the water surface. It was expected that the loss in sensitivity between any two injections was proportional to the alcohol vapor concentration of the previous run. A correction was then applied for the loss in sensitivity to each of the last six test runs. The amount of correction applied to each change in surface potential was based on the previous injection concentration and calculated so that the change in surface potential for the last injection when corrected was the same as the first. When the p. p. m. *versus* the corrected mv. change in surface potential was plotted on semilog paper, the points were found to fall very close to a straight line.

Another check on the exponential relationship for ethyl alcohol was made using ethyl alcohol vapor along with varied concentrations of other alcohol vapors. In this case, a series of distilled-water surfaces was used. For each test the surface sensitivity of the particular water surface was standardized by determining the response to ethyl alcohol vapor of known concentration. This was done using the exponential relationship based on the most often encountered sensitivity of a distilled-water surface to ethyl alcohol vapor. Following this, a different alcohol vapor was injected and the change in surface potential for this second injection was corrected for the particular surface sensitivity as previously determined using ethyl alcohol vapor. Repeated tests were made in this manner at each of several different concentrations of the nonethyl alcohol vapor, and good agreement was found in the mv. change in surface potential caused by a given vapor concentration for several different alcohols. This indicated that the exponential relationship provided the correct factor for adjusting the sensitivity of individual water surfaces over the range of vapor concentration used so that all surface potential changes could be corrected for any differences in individual water surfaces.

The agreement found in both of the above checks concerning the correctness of the exponential relationship for ethyl alcohol was considered well within the accuracy of the equipment. Errors of 3% were expected in the determination of the flow rate of the air stream along with errors of 1% in the volume of vapor displaced into the air stream and errors of 3% in the determination of the vapor pressure. Errors were also expected in reading the surface potential, in determining the total change in surface potential (due to corrections for drift and other disturbances in the potential), and in calculating corrections for loss in sensitivity of various surfaces (due to exposure and variations within the surfaces themselves). These latter errors were estimated to be as great as 5%. The total experimental error was considered to be within 10%.

As a result of these tests, the exponential relationship between ethyl alcohol vapor concentration in p. p. m. by volume in the air stream and the mv. change in surface potential was accepted for the range of vapor concentrations employed, and the practice of rating distilled-water surfaces on the basis of the ethyl alcohol vapor sensitivity was considered valid.

Normal Homologous Alcohol Vapors.—Butyl alcohol at various concentrations was used as a contaminant vapor, and the exponential relationship between p. p. m. and mv. change in surface potential was again indicated. It was noted that plots of p. p. m. *versus* mv. change on semilog paper for both ethyl and butyl alcohol had practically identical slopes. See Fig. 1. The straight line relationship on semilog paper was then assumed for the other members of the normal homologous alcohol series, and checks were made at two different vapor concentrations for each different alcohol. The tests at each vapor concentration were repeated a minimum of three times to check agreement and reliability of the data.

In all, five different members of the homologous alcohol series were tested in the manner described above using distilled-water surfaces. These were

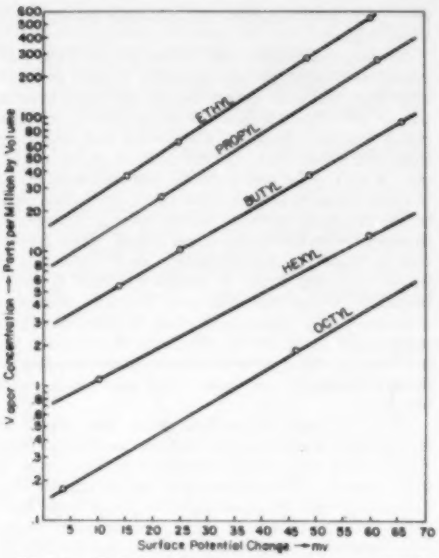

Fig. 1.—Relationship between surface potential change and vapor concentration for normal alcohol vapors.

ethyl, propyl, butyl, hexyl, and octyl alcohol having two, three, four, six, and eight carbon atoms, respectively. The straight line relationships between the logarithm of the vapor concentrations in p. p. m. by volume and surface potential changes in mv. for propyl, hexyl, and octyl alcohols were found, when plotted, to be very nearly parallel to the same lines plotted for ethyl and butyl alcohol vapors. The vapor pressure of ethyl alcohol is 64 mm. Hg at 27° while that of octyl alcohol is only 0.16 mm. Hg at 27°. This range in vapor pressure is greater than the maximum range over which saturated vapor could be injected into the air stream with the apparatus employed. The workable range for injecting saturated vapor into the air stream was from 0.015 to 0.25 cc. per second. This is a ratio of approximately 16:1 as compared with the ratio of 400:1 for the vapor pressures of ethyl and octyl alcohol at room temperature. Therefore it was not possible to inject vapors of all the different alcohols tested into the air stream at the same concentration in parts per million by volume. In order to increase the range over which comparisons could be made, some of the curves were slightly extended.

The data for ethyl, propyl, butyl, hexyl, and octyl alcohol vapors were considered and plotted in three different ways. The first was the relationship between the vapor concentration in the air stream in p. p. m. by volume and the mv. change in surface potential. The second was the relationship between the rate at which saturated vapor was injected into the air stream and the mv. change in surface potential. The third was the relationship between the vapor concentration in the air stream in p. p. m. by volume and the ratio of the mv. change in surface potential to the p. p. m. by volume vapor concentration.

The first relationship is shown in Fig. 1 where the logarithm of the vapor concentration in p. p. m. by volume is plotted against the change in surface potential. This figure indicates that, for a given change in the surface potential, the required p. p. m. decreased as longer-chain alcohol vapors were used. The lines for the various alcohols are nearly parallel and each is well separated from the next. The various lines could be represented by an exponential equation of the form $y = a(b)^x$, where y refers to the p. p. m. and x to the mv. change in surface potential. The value of b is approximately 1.0 in each case and the various values of a are: ethyl, 17.18; propyl, 6.76; butyl, 3.01; hexyl, 0.65; and octyl 0.15.

The second relationship, which was between the rate in ml. per second at which saturated vapor was injected into the air stream and the mv. change in surface potential, is shown in Fig. 2. The reason for the interest in this relationship was that many olfactory studies had been done with trained investigators sniffing equal amounts of saturated vapor. The curves appear to be logical in that repeated increases in the injection rate produced successively smaller increases in the mv. change of the surface potential. The curves generally indicate a smaller change in the surface potential for a given injection rate for each heavier alcohol vapor. The unusual feature of Fig. 2 is the location of the butyl line which indicates that butyl alcohol vapor caused a greater change in surface potential than either ethyl or propyl for a given injection rate of saturated vapor. This might be due to the greater solubility of ethyl and propyl alcohol in water which reduced the ability of these vapors to change the surface potential of the water.

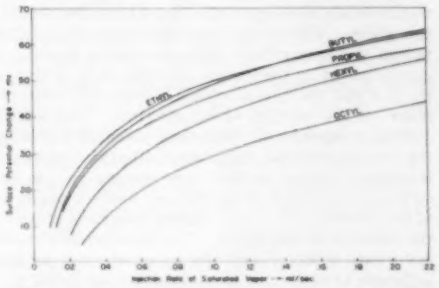

Fig. 2.—Relationship between surface potential change and injection rate of saturated vapor for normal alcohol vapors.

The third relationship considered was between the vapor concentration in the air stream in p. p. m. by volume and the ratio of the change in surface potential to the vapor concentration. This relationship for the various alcohols is shown in Fig. 3. The mv. to p. p. m. ratio[1] can be considered as a measure of the ability of a given alcohol vapor to change the surface potential upon adsorption, and the graph shows that this ability increases with increased chain length.

The hydroxyl group of the normal alcohol molecules is water attractive and thus it is this end of the

[1] This ratio has also been defined as surface sensitivity when the ratio is used to describe the sensitivities of various surfaces to a given contaminant vapor.

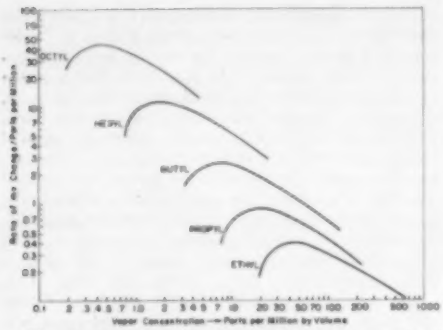

Fig. 3.—Relationship between vapor concentration and ratio of mv. change to p. p. m. for normal alcohol vapors.

chain which attaches itself to the water surface (3). Such groups attached to a hydrocarbon-chain might be called water-soluble groups, and in the shorter-chain compounds, such as ethyl alcohol, the water-soluble hydroxyl group confers solubility on the whole molecule. In the long-chain compounds, such as the heavier alcohols, this group cannot pull the whole molecule into the water owing to the resistance of the long-chains to immersion, and thus the adsorbed layer is made up of molecules with the water-soluble group down close to the water and the hydrocarbon-chain extending away from the surface. The extent of the attraction for water of the water-soluble groups can be roughly determined from the length of the carbon-chain required to prevent solution of the molecule.

Figures 1 and 2 indicate that, for a given concentration of alcohol vapor, the longer the chain length, the greater the change in surface potential. The reason the longer-chain alcohols appear to have a greater effect on the surface potential may be due to the fact that they are insoluble in water and must remain on the surface when adsorbed. The nearly complete recovery of the surface potential following the termination of an injection of ethyl alcohol vapor into the air stream probably was due not only to desorption from the surface but also to the movement of the adsorbed alcohol molecules from the surface to the interior of the liquid. The amount of alcohol going into solution with the water was not judged sufficient to cause any permanent change in the surface potential. The changes in surface potential caused by the different alcohol vapors most likely were due to the vertical component of the dipole of the adsorbed molecules with any reorientation of the water molecules playing a minor role. Thus the difference in the ability between short- and long-chain alcohol molecules to change the surface potential of a water surface may be mostly due to orientation of the adsorbed molecules on the surface, with the longer-chain molecules in better alignment.

OLFACTORY COMPARISONS

An interesting comparison can be made between the data secured using the normal homologous alcohol vapors and data obtained by other investigators who studied the olfactory properties of the same alcohol vapors. Various homologous series, and in particular the alcohols, have been investigated in recent years with regard to the olfactory intensities of these vapors.

Kruger, Feldzamen, and Miles (4) of Yale Univerity used three people especially trained in olfactory observation to determine the olfactory intensities of ten aliphatic alcohols from 3 to 12 carbon-chain lengths. Some of the stated conclusions of their study were that: (*a*) the alcohols which are soluble in both polar and nonpolar solvents were found to have the more intense odors; and, (*b*) within most of the range of alcohols tested, olfactory intensity decreases with increasing chain length unless correction is made for the different vapor pressures, in which case the trend is reversed. This is interpreted to imply that olfactory intensity decreases with increasing chain length when the alcohol vapors were tested using specific dilutions of saturated vapor, but for equal molecular concentrations, the olfactory intensity increased with increasing chain length.

The conclusion of the Yale group with regard to chain length is analogous to the relationship between chain length and surface potential change found in this research. Comparisons of the various alcohol vapors in terms of equal p. p. m. by volume indicates that the change in surface potential increases with increasing chain length, as shown in Fig. 1. It is also interesting to make a comparison between the two different studies in terms of equal volumes of saturated vapor. In Fig. 4 the change in surface potential is plotted against chain length for the specific injection rate of 0.2 ml. per second of saturated vapor. This is similar to a plot of odor intensity *versus* chain length as determined by each of the three "sniffers" used by the Yale group. In general, the "sniffers" rated the alcohols of 4, 5, and 6 carbon members slightly more intense than either the lighter or heavier alcohol vapors; however, the individuality of human beings was also apparent.

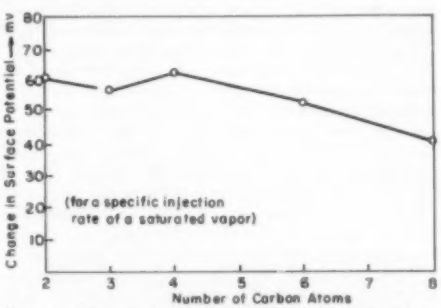

Fig. 4.—Change in surface potential *versus* chain-length for normal alcohol vapors.

The olfactory properties of the normal homologous alcohols were also studied by Dethier and Yost (5) of Johns Hopkins University. This group experimented with blowflies to make use of several advantages offered by insects as compared to man for this type of study. The general procedure used by them was to pass two air streams with little or no mixing through a cage containing the flies and to increase the alcohol vapor concentration in one air stream until the flies rejected it in preference to the other. They reported that when rejection thresh-

olds of the normal alcohols were expressed as molar concentrations and plotted against their respective chain lengths on logarithmic coordinates, the trend toward linearity was at once apparent.

Using the data secured in this investigation and arbitrarily taking a 25 mv. change in the surface potential as a critical or rejection figure, a graph as shown in Fig. 5 was obtained. The logarithmic coordinates are chain length and the alcohol concentration in p. p. m. by volume which produced the 25 mv. change in surface potential. The relationship between the logarithm of the vapor concentration and the logarithm of the chain length is clearly linear, and comparison of this relationship with the relationship reported by Dethier and Yost indicates a definite similarity.

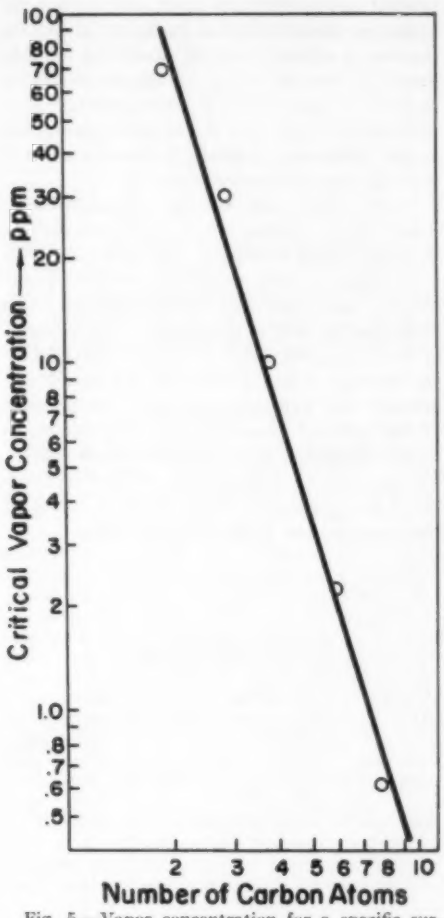

Fig. 5.—Vapor concentration for a specific surface potential change *versus* chain-length for normal alcohol vapors.

SUMMARY

The general results of this study are:

1. Equal concentrations of different vapors of a homologous alcohol series in an air stream, measured in parts per million by volume, cause different changes in the surface potential of a distilled-water surface.

2. The presence of alcohol vapors in an air stream at concentrations of the same order of magnitude as the minimum concentrations detectable by the human nose can be determined by measurement of surface potential change.

3. Relationships between changes in surface potential and chain length of the normal homologous alcohols are analogous in certain respects to reported relationships between olfactory stimulation and chain length of the same vapors.

CONCLUSIONS

There appear to be several factors associated with the surface potential change of a water surface caused by adsorption which may be advantageous as far as the detection of odorous materials in the atmosphere is concerned. These are: (*a*) the facility with which a liquid can be employed; (*b*) the capability of a liquid to act as a solvent—as solubility has often been associated with odor (6); and (*c*) the orientation freedom of both the liquid and adsorbate molecules. These factors allow the adsorption and surface potential change processes of a liquid surface to be more readily influenced by the architectural arrangement of the adsorbate molecule, a factor generally considered in the study of odor. The results of this investigation indicate certain analogies between the changes in surface potential and the olfactory stimulation produced by certain vapors but should not be interpreted to imply that odor has been measured by electrical-mechanical means. The results do indicate the desirability of further research regarding surface phenomena with the goal of learning more about the roles played by the various physical properties of the adsorbate molecules.

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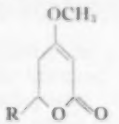
The Reaction of Δ^2-Cyclohexenone With Trifluoroperoxyacetic Acid*

By EDWARD E. SMISSMAN and FRED B. BLOCK

The reaction of 2-cyclohexenone with trifluoroperoxyacetic acid gave 2-hydroxyadipic acid as a final product. The failure of 2-cyclohexenone to yield Δ^2-caprolactone or 2,3-dihydroxycaprolactone demonstrates the favored transition state involves carbon-carbon double bond participation and not alkyl group migration.

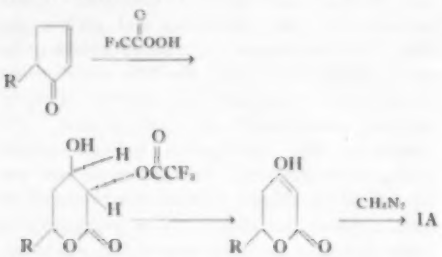
THE ONLY reported synthesis of dihydrokavain [6-(β-phenylethyl)-5,6- dihydro - 4 - methoxy-2-pyrone], IA, a constituent of *Piper methysticum*, is the reduction of the closely related compound kavain IB, (6-styryl-5,6-dihydro-4-methoxy-2-pyrone) (1,2).



I

(*A*) R = $C_6H_5CH_2CH_2$—
(*B*) R = C_6H_5CH=CH—

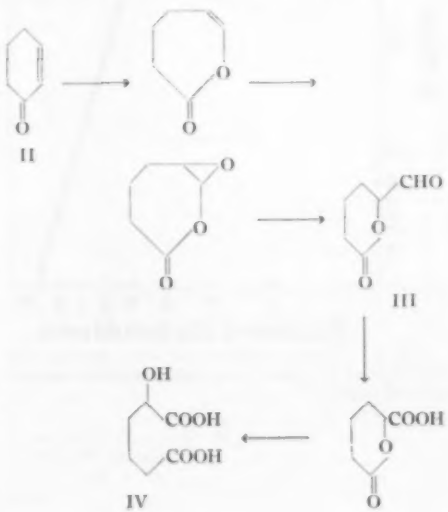
A possible synthetic route to dihydrokavain, utilizing the Baeyer-Villiger reaction can be outlined as follows:



The Baeyer-Villiger lactonization and hydroxylation step appeared to be of particular interest, as this one-step combination of reactions had not been reported. A good method for preparing 5-alkyl-2-cyclopentenones, as well as 2-cyclopentenone itself, could not be found and rather than attack this portion of the problem, it was decided to use 2-cyclohexenone, II, as the model compound to test the practicality of the proposed reaction.

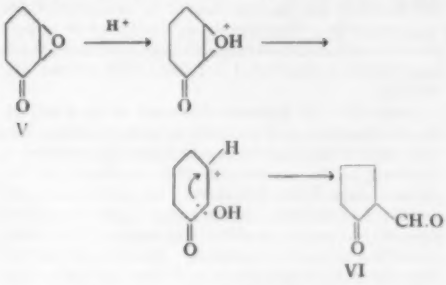
Peroxytrifluoroacetic acid was selected as the reagent for the rearrangement-hydroxylation step because of high yields reported in the preparation of lactones (3) from cyclohexanone and cyclopentanone (71 and 84 per cent, respectively) and the hydroxylation of normally resistant acrylates and methacrylates in good yield by this reagent (4). The reaction procedure utilized gave an amount of nonvolatile material in excess of that expected for the peracid oxidation. The infrared absorption spectrum of the reaction mixture at this point indicated the possible presence of a trifluoroacetyl group, 5.59 μ (5). The product was treated with 3 per cent methanolic hydrogen chloride to effect methanolysis, and on removal of solvent a red oil exhibiting infrared bands at 5.59 and 5.76 μ, in addition to a band at 2.84 μ, was obtained. The presence of a moderately strong band at 5.59 μ was unexpected as this band was originally believed associated with the trifluoroacetoxy group.

Vapor phase and column chromatography would not resolve this red oil and it was decided to make a solid derivative. On treatment with *p*-toluidine, a white crystalline compound was obtained and had the correct analysis for a hydroxyadipic acid di-*p*-toluidide. The presence of the adipic acid skeleton was demonstrated by the isolation of adipic acid after the red oil was refluxed with hydriodic acid, while the presence of the hydroxyl group was positively confirmed by the conversion of the di-*p*-toluidide derivative to its acetate. The location of the hydroxy group was suggested by the apparent course of the reaction to be in the 2-position, IV.



* Received April 3, 1959, from the University of Wisconsin, School of Pharmacy, Madison 6.

Synthetic 2-hydroxy adipic acid was prepared by an independent method and its di-*p*-toluidide was identical with the material obtained from the reaction mixture. A small amount of a 2,4-dinitrophenylhydrazone could be obtained from the reaction mixture and it corresponded to 5-formyl-5-pentanolid, III. Work by House and Wasson (6) on the rearrangement of α,β-epoxycyclohexanone has demonstrated that under acidic conditions 2,3-epoxycyclohexanone, V, rearranges with ring contraction to the keto



V VI

aldehyde, VI. The failure of 2-cyclohexenone on peroxidation to yield Δ^2-caprolactone or 2,3-dihydroxy caprolactone demonstrates the transition state favored in this compound involves carbon-carbon double bond participation and not alkyl group migration.

EXPERIMENTAL

Reaction of 2-Cyclohexenone with Peroxytrifluoroacetic Acid.—Seventy-five milliliters of dry methylene chloride and 11.8 Gm. (0.312 mole) of 90% hydrogen peroxide were placed in a flask equipped with a thermometer, dropping funnel condenser, and magnetic stirrer. Over a period of one hour, 78.5 Gm. (0.374 mole) of trifluoroacetic anhydride was added to the rapidly stirred mixture which was cooled in an ice bath. Additional stirring was continued for fifteen minutes. To the cooled (10° or below) peroxytrifluoroacetic acid was added 10.0 Gm. (0.104 mole) of 2-cyclohexenone, dissolved in 25 ml. of dry methylene chloride, over a period of one and one-half hours. After addition was complete the red solution was allowed to warm to room temperature and then refluxed for two hours. The volatile solvents were removed under reduced pressure.

The liquid residue (24.3 Gm.) was refluxed with 300 ml. of 3% methanolic hydrogen chloride for three hours and then allowed to stand eight hours at room temperature. The volatile material was removed *in vacuo* leaving 15.5 Gm. of liquid residue which had an infrared spectrum showing bands at 2.84, 5.59, and 5.76 μ; ultraviolet spectrum showed only end absorption.

Isolation of Adipic Acid. To 0.947 Gm. (0.005 mole based on dimethyl 2-hydroxy adipate) of liquid residue, under a constant stream of dry nitrogen, was added 17 Gm. of freshly distilled 57% hydriodic acid. The mixture was refluxed one hour and then concentrated to a small volume. The mixture was dissolved in ether, washed with sulfurous acid, and dried. A 66.6% yield, 0.488 Gm. (0.0033 mole) of adipic acid was obtained, m. p. 150.7–152°.

2-Hydroxy Adipic Acid Di-*p*-toluidide.—A crystalline derivative was prepared by combining 0.202 Gm. (0.001 mole based on dimethyl-2-hydroxyadipate) of the liquid residue with 0.814 Gm. (0.0076 mole) of *p*-toluidine and heating the mixture at 190–200° for two hours. After cooling and washing with ether, 2-hydroxyadipic acid di-*p*-toluidide, m. p. 183.5–188.5° was obtained. It was recrystallized from ethanol-water, m. p. 187.4–187.9°.

Anal.—Calcd. for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.65; H, 7.23; N, 8.03.

2-Acetoxyadipic Acid Di-*p*-toluidide. Forty-five milligrams of the di-*p*-toluidide of 2-hydroxyadipic acid was dissolved in 0.7 ml. of pyridine and 0.5 ml. of acetic anhydride was added; the mixture was heated for one hour. The excess reagents were removed *in vacuo* leaving a solid product which was recrystallized from ethanol-water, m. p. 172.4–173.2°.

Anal.—Calcd. for $C_{22}H_{26}N_2O_4$: C, 69.09; H, 6.85; N, 7.33; acetyl, 11.25. Found: C, 69.19; H, 6.56; N, 7.26; acetyl, 13.3.

2-Hydroxyadipic Acid.—Dimethyl-2-chloroadipate, b. p. 145–150°/15 min., N_D^{20} 1.4505, (lit., b. p. 138–139°/10 mm., N_D^{20} 1.4510) (7) was prepared by the method of Triebs and Holbe.

One gram of dimethyl-2-chloroadipate was treated with 13 ml. of 10% aqueous methanolic sodium hydroxide. After three and one-half hours of refluxing, the mixture was cooled, acidified with concentrated hydrochloric acid, and evaporated to dryness. The solid residue was boiled with ether and the ether solution evaporated to give a colorless oil which crystallized on standing. This material was converted to the di-*p*-toluidide, m. p. 170.8–172.5°. Mixed melting points with previously described samples gave no depression.

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16α-Hydroxy Steroids II*

Partition Chromatography of Triamcinolone and Related Steroids

By LELAND L. SMITH, THEODORE FOELL, RALPH DE MAIO, and MURRAY HALWER

Paper chromatographic systems and column partition systems suitable for analyzing triamcinolone in pharmaceutical preparations and in extracts of biological samples are described. Comparative mobilities of related steroids are reported for the several systems. Both qualitative and quantitative aspects of the chromatographic operations are described.

THE CHARACTERIZATION of the corticosteroid hormone triamcinolone (9α-fluoro-11β,16α,-17α,21 - tetrahydroxy - 1,4 - pregnadiene - 3, 20-dione) by instrumental means has been reported in the first paper of this series (1). For analyses involving more complex situations as exist in metabolic studies of the drug, preliminary chromatographic separations become necessary. The several paper chromatographic systems and column partition systems which have been of use in routine examination of triamcinolone and related steroids are presented in this paper.

PAPER CHROMATOGRAPHY, QUALITATIVE

Recently reports have been published covering the use of Zaffaroni-type paper chromatographic systems for the separation of more polar steroids (2), which systems together with the chloroform/formamide system of Schindler and Reichstein (3) afford separation of a variety of more polar steroids. The application of Zaffaroni-type systems to triamcinolone and related 16α-hydroxylated steroids has been described (4). Although some Bush-type systems capable of rapid separations of more polar steroids have been described (5), no systematic examination of very polar steroids in Bush-type systems has been reported. In our hands modified Bush-type systems have been found to be more versatile in the complex separations involved in the production and analysis of triamcinolone than have the Zaffaroni-type systems.[1]

For the routine examination of triamcinolone samples derived from a variety of sources the following conditions must be met: (*a*) that the $\Delta^{1,4}$-3-ketosteroids be well separated from their respective Δ^{4}-3-ketosteroid analogs, (*b*) that the respective rearrangement product of each 16α-hydroxylated steroid be well separated from the parent steroid, and (*c*) that running time be limited to no longer than overnight. The six solvent systems used are described in the experimental part. Where possible the systems have been adjusted to afford mobilities for at least one steroid component of interest in the vicinity of R_f 0.5. A more stringent requirement met by systems I and II for the free steroid alcohols is of importance, namely that the rearrangement product 1,2-dihydrotriamcinolone isomer be separated from triamcinolone. The fortuitous similarity of papergram mobilities in several Bush-type systems of these two steroids is illustrated in Table I with systems III and IV.

Using the six systems described in descending chromatography it is possible to separate many derivatives of triamcinolone and many intermediates involved in the several reported syntheses (6, 7). Examination of the free steroid alcohols is made in systems I through IV, with system II being used for general qualitative examinations, system I run overnight for quantitative analysis. Systems III and IV serve for both free alcohols and their acetates, etc., while systems V and VI are for the acetates and acetonide derivatives.

An arbitrary equilibration period of two hours is used for each papergram system, and the tanks are maintained at about 29° in a closed room. Although higher temperatures and longer equilibration periods have been examined, the short running time and the lower temperature afford useful chromatographic separations. The 29° temperature is maintained by one 100-watt incandescent lamp overhead in a small tank room. Using these conditions the data of Table I were collected. Although it is possible to obtain reproducible R_f values for these steroids, standard steroid markers are run with each set of samples, as several factors influence mobility under the described conditions.

It is of particular importance in the case of system II that attention be paid to the variability in R_f values. This system is of great value in examination of triamcinolone samples; however, the mobility of a steroid is dependent on the actual running time. Thus, at one hour lower R_f values are obtained for all steroids studied than at the standard four hour running time. Also the solvent front migration rate decreases with increasing running time, covering 17.5 cm. in the first hour, 14.3 cm. in the second hour, and 11.0 cm. in the fourth hour. Freshly prepared tanks yield mobility values as presented in Table I; however, as the tanks are used, slight losses in solvent plus other effects cause a decrease in mobility of all steroids, so that after several weeks of use, lower R_f values are generally obtained. Despite these several deficiencies, system II is of very great value in studies of triamcinolone chemistry and biochemistry. It is possible that the characteristics mentioned obtain with other Bush systems described in the literature.

The detection of the steroids on papergrams is accomplished with several techniques. The ultraviolet absorption properties of these steroids affords a ready means of location using photographic meth-

* Received May 20, 1959, from the Chemical Process Improvement Dept., Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.

[1] The mobility of triamcinolone in the chloroform/formamide (50% methanolic formamide impregnation) system is: 3.1 cm./22 hr.; of 1,2-dihydrotriamcinolone, 3.5 cm./22 hr.; of 9α-fluorohydrocortisone, 20 cm./22 hr.

TABLE I.—PAPERGRAM CHARACTERIZATION OF TRIAMCINOLONE AND RELATED STEROIDS

Steroid	R_f of Free Alcohol				R_f of Acetates, Diacetates			R_f of 16α, 17α-Acetonide		
	I	II	III	IV	IV	V	VI	IV	V	VI
Triamcinolone	0.18	0.42	0.14	0.13	0.89	0.27	0.12	0.88	0.36	0.13
1,2-Dihydrotriamcinolone	0.24	0.52	0.22	0.19	0.92	0.40	0.22	0.92	0.46	0.25
9α-Fluoroprednisolone	0.37	0.67	0.37	..	0.89	0.30	0.12	..	..	..
9α-Fluorohydrocortisone	0.45	0.80	0.47	0.59	0.90	0.35	0.18	..	..	..
Triamcinolone isomer	0.10	0.22	0.08	0.06	0.76	0.08	0.02	0.62[a]	0.10[a]	0.03[a]
1,2-Dihydrotriamcinolone isomer	0.12	0.30	0.13	0.10	0.85	0.18	0.06	..	0.16[a]	0.06[a]
16α-Hydroxyprednisolone	..	0.44	..	0.23	..	..	..	..	0.44	0.29
16α-Hydroxyhydrocortisone	..	0.49	..	0.30	..	..	..	..	0.57	0.40
Prednisolone	0.53	0.73	0.46	0.44	0.89	0.30	0.15	..	..	..
Hydrocortisone	0.58	0.75	0.53	0.53	0.93	0.46	..	..	..	..
Prednisone	0.69	0.79	0.58	0.66	..	0.60	..	..	..	..
Cortisone	0.73	0.81	0.65	0.69	..	0.66	0.43	..	..	..

[a] The acetonides are in all probability 16α, 17aα-cyclic acetonides of the D-homo-16α,17aα-dihydroxy-17aβ-hydroxymethyl-17-ketones and not simple 16α,17α-acetonides.

ods (8) or using fluorescent screens (9). The reducing properties of triamcinolone permit facile detection using tetrazolium salts, or Tollen's reagent.

The failure of triamcinolone and related $\Delta^{1,4}$-3-ketosteroids to give the alkaline fluorescence test of Bush (10) which is specific for Δ^4-3-ketones, allows direct differentiation of these types. Although *p*-phenylenediamine phthalate (11), 2,4-dinitrophenylhydrazine (12), and salicyloyl hydrazide (13) have been variously reported as color tests for $\Delta^{1,4}$-3-ketosteroids, in our hands these tests have not been of use with triamcinolone and other common $\Delta^{1,4}$-3-ketones. However, isonicotinic acid hydrazide has proven an excellent method for the detection of both $\Delta^{1,4}$-3-ketosteroids and Δ^4-3-ketosteroids on papergrams (14).

Our routine examination of paper chromatograms involves five steps: (*a*) examination under short wavelength ultraviolet light using a modified Haines-Drake scanner (9) as described by von Arx and Neher (15), (*b*) examination under long wavelength ultraviolet light (365 mμ) for fluorescent components, (*c*) application of the weak ethanolic isonicotinic acid hydrazide reagent of Smith and Foell (14) for the detection of Δ^4-3-ketosteroids, followed by (*d*) application of the strong methanolic isonicotinic acid hydrazide reagent for $\Delta^{1,4}$-3-ketones, and finally (*e*) alkaline tetrazolium blue spray for reducing α-ketols. The specificity conferred by this series of tests enables an experienced operator to establish the probable steroid nature of an unknown component, and together with relative mobility measurements, to recognize a large variety of metabolites and alteration products of triamcinolone and related steroids.

Experimental

The paper partition systems are prepared in the usual manner (10) by equilibrating appropriate volumes of the solvents of choice for several days at the temperature at which the solvent mixture will be used. In practice, the mixtures are kept in the same room as the chromatographic jars and the phases are separated a short time before the chromatographic run is to be started. The upper (organic) phase is used for irrigation of the papers (mobile phase) in all systems. The compositions of the solvent systems used are as follows:

System I: benzene/ethanol/water, 2/1/1. Equilibration time, two hours, running time, sixteen hours. (R_f data were taken on the system run for four hours.) For free steroid alcohols.

System II: benzene/acetone/water, 2/1/2. Equilibration time, two hours, running time, four hours. For free steroid alcohols.

System III: benzene/dioxane/water/acetic acid, 4/1/2/1. Equilibration time, two hours, running time, five hours. For free alcohols and acetates.[2]

System IV: benzene/ethanol/water, 2/1/2. Equilibration time, two hours, running time, five hours. For free alcohols and acetates.

System V: toluene/petroleum ether (b.p. 30–60°)/methanol/water, 12/8/13/7. Equilibration time, two hours, running time, two and one-half hours. For acetates, acetonides, etc.

System VI: benzene/petroleum ether (b. p. 90–100°)/methanol/water, 5/5/7/3. Equilibration time, two hours, running time, three and one-half hours. For acetates, acetonides, etc.

These solvent systems were all run in the descending technique, using Whatman No. 1 filter paper.

PAPER CHROMATOGRAPHY, QUANTITATIVE

For quantitative analysis the separated components are eluted and ultraviolet absorption measurements are made on the eluates. System I is recommended for this work, as the system has the designed advantage of running overnight so sample preparation, equilibration, etc., may occur in the afternoon of one day and location, elution, and spectrophotometric analysis be completed the next day.

Experimental

Preparation of Tanks.—Glass cylinders 12 inches in diameter, 24 inches high are lined with Whatman No. 1 filter paper, the liners dipping into the stationary phase in the bottom of the tank. A dish of mobile phase is placed in the bottom of the tank and a filter paper wick is suspended from the solvent trough apparatus in the center of the tank so that the wick dips into the dish of mobile phase. Lids are ground to fit each tank individually and are held solvent tight by plastic tubing rings filled with lead shot. The lids are not lubricated. After forty-eight hours of equilibration at 29° the tank is ready for use.

[2] This solvent system was suggested by Mr. R. H. Blank of these laboratories.

Preparation of Papers.—Whatman No. 1 filter paper (18.25 × 22.5 in.) is washed chromatographically with ethanol for seventy-two hours. After the papers are washed they are handled only with tissue papers. The dried (hood) papers are cut into sheets 9.5 cm. wide, a starting line is ruled across the paper four inches from one end and the other end is serrated with pinking shears to allow free runoff of excess solvent, and the papers are stored in filter paper wrappers until used.

Sample Preparation.—The steroid sample is weighed on a Sartorius Micro Torsion Balance, Model MTB-1, to the nearest 0.02 mg. Generally a 1 mg./ml. solution is prepared in distilled methanol. Application of the sample is made via a 100 µl. micropipet calibrated "to contain" (a later improvement was made when "to deliver" pipets became available commercially). Care is taken that the steroid solution is not drawn above the calibration mark. Application of the solution to the paper is made by streaking across the paper; the streak is begun about 1 cm. from one edge and terminated about 1 cm. from the opposite edge, such application resulting in a decreased "edge" effect in the band movements. The "to contain" pipet is then washed out with 100 µl. of methanol, the washings being applied to the paper in a similar manner. Suitable drying time is allowed between applications. Samples are prepared in duplicate, using the same pipets and solutions. The prepared papers are then hung in the chromatographic tank for equilibration.

Chromatographic Development. — Chromatographic tanks for quantitative papergram assay are kept separated from tanks prepared in an identical manner but used for qualitative papergram examinations. The tanks prepared using system I are equilibrated for two hours after the prepared papers are placed in them. The running time (sixteen hours) is the same as used for qualitative examination, unless some of the more mobile components (such as 9α-fluorohydrocortisone) are to be assayed, in which case suitable shorter times are required. Blank papergrams containing no steroid but otherwise handled in exactly the same way as the sample sheets are run with each tank used; thus three sample sheets and one blank sheet are generally run together in one tank. The developed papers are dried in air for thirty minutes, viewed rapidly under the modified Haines-Drake ultraviolet scanner, and the steroid-absorbing zones marked in pencil. In order to see steroids on the papergram with this technique at least 8–10 µg. of steroid is required. Where the amount of steroid per zone is less than this limit it is necessary to determine the position which would be occupied by the steroid sought from relative mobility measurements. Our practice has been to measure all mobilities in terms of triamcinolone as unit mobility (R_s 1.00); thus triamcinolone isomer has R_s 0.54; 1,2-dihydrotriamcinolone R_s 1.30; 1,2-dihydrotriamcinolone isomer, R_s 0.74, etc. The zones located via ultraviolet absorption are cut out; the zones determined by calculation are cut out 1.5 in. wide. The pieces are warmed with 10 ml. of absolute ethanol for one hour at 60° in closed tubes, cooled to room temperature, and the absorbance of the solutions determined (after centrifugation, if necessary to remove lint) at 239 mµ read against their appropriate blanks treated similarly.

The blank sheet is cut into zones exactly according to the zones found for the steroids being assayed, eluted in the same way, and read against absolute ethanol as a check on the operation, and then used as the blank solution for the appropriate steroid determination. The blank absorbance values are of the order 0.002 to 0.03 units *versus* absolute ethanol.

Calculation of Results.—A spectrophotometric factor (µg./ml. divided by absorbance at 239 mµ) is determined on a pure steroid sample (not chromatographed). The absorbance of the unknown eluate times the factor times the eluate volume gives the micrograms of steroid found, uncorrected for recovery. The factors used are: triamcinolone, 26.8; 1,2-dihydrotriamcinolone, 23.7; triamcinolone isomer, 26.8; 1,2-dihydrotriamcinolone isomer, 23.7; 9α-fluorohydrocortisone, 24.9.

In a series of experiments with pure steroids an average recovery of 80 µg. out of 100 µg. applied to the paper was found. The factor was the same over the range 25–100 µg. of sample applied, although at levels less than 25 µg. somewhat less (*ca.* 70%) was found. Individual recovery factors on pure samples were often as high as 90%. Recovery from mixtures of several components was not affected by the nature of the mixture. The recovery factor should be determined under the conditions used and applied to the uncorrected data.

Alternate treatment in the form of calibration curves constructed over the range 0–100 µg. of steroid applied *versus* µg. of steroid recovered yield linear relationships, although irregularities occur in individual cases. Attempts to rely on the absolute weight of steroid found for the calculation of composition were not pleasing, so sample composition is expressed in terms of per cent of total steroids found, with the actual weight found also reported. Where material balances for multicomponent mixtures fell much below the 80% factor the assay is suspect.

Some representative analyses are presented in Table II. All samples are run in duplicate and are so reported.

TABLE II.—REPRESENTATIVE DUPLICATE ANALYSES ON TRIAMCINOLONE PROCESS SAMPLES

Sample No.	Steroid Found: Triamcinolone µg.[a]	Triamcinolone %[b]	Triamcinolone Isomer µg.[a]	Triamcinolone Isomer %[b]	1,2-Dihydrotriamcinolone µg.[a]	1,2-Dihydrotriamcinolone %[b]
1	82.5	98.4	1.4	1.6	0	0
	83.9	100.0	0	0	0	0
2	79.1	97.5	1.1	1.4	0.9	1.1
	80.7	94.7	2.1	2.5	2.4	2.8
3	71.0	94.5	2.7	3.6	1.4	1.9
	71.0	93.7	2.9	3.8	1.9	2.5
4	84.1	94.1	0.8	0.9	4.5	5.0
	83.4	93.4	1.3	1.5	4.5	5.1
5	45.4	50.1	45.1	49.9	0	0
	44.3	49.5	45.1	50.5	0	0

[a] µg. found, uncorrected for recovery.
[b] Per cent of total spectrophotometric activity found.

COLUMN PARTITION CHROMATOGRAPHY

For such purposes as final analysis of steroid samples for commercial sale the quantitative analytical methods involving paper chromatography often are unsatisfactory and methods employing column

chromatography are used. The increased sample charge permits more precision and accuracy in the measurement of minor contaminants, indeed, permits multiple analyses to be made on each resolved fraction. Although somewhat lower resolving power is usually had in comparison with paper chromatographic separations using the same solvent mixtures, the advantages of rapid operation, increased by the use of air pressure, together with the possibility of application of several specific methods to the eluates and increased precision, etc., makes this technique invaluable where several closely related steroidal impurities must be controlled.

A partition system devised by Dr. N. Rigler, of these laboratories, comprised of dioxane/cyclohexane/water in varying proportions, has proved of analytical as well as of preparative value. According to the degree of resolution desired the solvent ratios of 5/2/1, 5/3/1, and 5/4/1 may be used. Characteristic hold-back or retention volumes permit rapid recognition of the identity of the steroids present in a given sample, and by the use of modern detecting devices, ultraviolet absorbing fractions may be collected separately one from the other and analyzed according to the need.

Experimental

Preparation of Solvent.—1,4-Dioxane should be freshly distilled from over potassium hydroxide pellets. Reagent grade cyclohexane is used without prior distillation. The solvents, dioxane/cyclohexane/water, are mixed in the proportions 5/2/1, 5/3/1, or 5/4/1 according to the degree of separation required, thoroughly equilibrated at the temperature at which the column is to be run (room temperature), and allowed to separate.

Preparation of Column.—Celite 545 diatomaceous earth is washed with methanol and with ethyl acetate to free it of impurities absorbing in the 240 mμ region. To 4.5 Gm. of the washed Celite diatomaceous earth is added 1.9 ml. of the separated lower phase of the solvent system to be used, and thorough mixing of the solvent throughout the support is effected with a spatula. The moistened support is packed into a glass column, 1 cm. in inside diameter and 35 cm. long (exclusive of a 125-ml. reservoir at the top), in layers of about 0.5 Gm. Each increment is packed uniformly by repeated light strokes with a ramrod whose diameter is just slightly less than that of the column. Uniform packing is essential for proper resolution and for reproducible hold-back volumes.

Sample Preparation.—Steroid samples from fermentation broths, etc., by extraction with ethyl acetate or other appropriate solvent are evaporated to dryness without heat and the residue is dissolved in the lower phase of the solvent mixture in the same manner as for crystalline samples. Sufficient broth extract must be taken to afford about 3 mg. of total steroids. Approximately 3 mg. of the steroid sample is dissolved in 0.23 ml. of the lower phase of the appropriate solvent mixture and thoroughly mixed with 0.45 Gm. of washed Celite 545 diatomaceous earth support. The mixture is packed on top of the prepared column. Ten milliliters of the upper phase of the solvent mixture is used to rinse the dish in which the sample has been prepared and the rinse is added to the top of the packed column and the liquid level is marked. Air or nitrogen pressure is applied at 3–5 lb./sq. in. until the first drop of eluate appears. The level of the liquid is again noted. The volume difference between the two marks is the hold-back volume.

About 70 ml. of the upper phase of the solvent mixture is added to the column reservoir and gas pressure is reapplied and pressure is adjusted so that the eluate flows from the column at about 1 ml./min. The eluate passes through a trap, the purpose of which is to prevent air bubbles and separated lower phase from entering the flow cell. A hypodermic syringe is used to withdraw lower phase from the trap if the level builds up too high. From the trap the eluate flows into a spectrophotometric flow cell attached to a Beckman model DU spectrophotometer set for determination of absorbance at 240 mμ. The resultant photo-current is recorded on a suitable recorder.

After passing through the detection device the eluate is collected in a series of graduated cylinders. According to the shape of the curve being recorded on the recorder various fractions are taken and the elution process is continued until no further ultraviolet-absorbing materials pass through the flow cell. Each component is characterized by a hold-back volume number, that is, the ratio of the total volume of eluate collected up to the midpoint of the peak as charted on the recorder to the hold-back volume as determined initially. Representative hold-back volume numbers (range of values) are tabulated in Table III for triamcinolone and some related steroids.

TABLE III.—REPRESENTATIVE NUMBERS OF HOLD-BACK VOLUMES OF TRIAMCINOLONE AND RELATED STEROIDS ON PARTITION COLUMNS

Steroid	Hold-Back Volumse in System: Dioxane/Cyclohexane/Water Ratio 5:2:1	5:3:1	5:4:1
Triamcinolone	3.2–3.7	4.3–7.2	*ca.* 10
1,2-Dihydrotriamcinolone	1.5–2.5	2.6–4.4	6
Triamcinolone isomer	4.7–5.2	8.1–12.0	*ca.* 12
1,2-Dihydrotriamcinolone isomer	3.0–3.5	7.2	9
9α-Fluorohydrocortisone	0.6–1.4	2.1	..
9α-Fluoroprednisolone	1.7	2.6	..

The several fractions taken according to the shape of the elution curve may be analyzed along any of the lines already described (1). For polarography the fraction is evaporated to dryness, care being taken to remove the last traces of solvent prior to dissolving in the polarographic solvent. Polarographic determination is then made in the usual manner (1). Colorimetric determination with tetrazolium blue is performed in the same way as previously described, care being exercised in the evaporation of the solvent to avoid heating. Each fraction from the column may be examined by paper chromatography for further evidence of its homogeneity and identity.

A process sample of triamcinolone analyzed with this method gave: triamcinolone, 95.0%; triamcinolone isomer, 2.1%; 1,2-dihydrotriamcinolone, 1.1%, with the total material balance (polarographic)

98.2%. The recovery of steroids polarographically in the eluted fractions averaged 96.8% in a series of 25 triamcinolone process samples, with a range of 89.7–102.4%.

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Enzymatic Decomposition of Digitalis Glycosides IV*

By OLE GISVOLD

IN A PREVIOUS PUBLICATION the glycosides found in the first year's growth of a number of species of digitalis have been reported (1). The glycosides were identified via paper chromatography using two very useful, dependable, quite temperature-insensitive solvent systems. In some cases the major glycoside was isolated in a crystalline state. Some of these plants survived the winter and bloomed in the summer of 1958. This permitted us to identify more accurately the individual species. It also gave me an opportunity to subject their leaves to the qualitative identification of their major glycosides. Extracts of the fresh leaves obtained by enzyme favoring and enzyme inhibiting techniques were examined by paper chromatographic techniques previously described (1). In all cases the same results were obtained as with the leaves from the first year's growth, i. e., the same desglucoglycosides were detected when enzyme favoring conditions were used and the same native glycosides when enzyme inhibiting techniques were employed. The species that were investigated were *Digitalis mertonensis*, *Digitalis amandiana*, *Digitalis siberica*, *Digitalis grandiflora*, and *Digitalis orientalis*. In the case of *Digitalis orientalis* the detectable desglucoglycosides were digoxin and acetyl digitoxin and in the case of *Digitalis grandiflora* they were digoxin, digitoxin, and acetyl digitoxin.

A previous investigation (2) showed that when *Digitalis purpurea* was dried rapidly below 60° larger amounts of the native glycosides could be detected. It was further demonstrated that these drying conditions did not inactivate the enzyme that removes the terminal glucose residue of the native glycosides. This discovery prompted a similar investigation of the dried leaves of the first year's growth of some of the other species of digitalis and analogous results were obtained when enzyme favoring and enzyme inhibiting techniques were employed. The same desglucoglycosides were detected when enzyme favoring conditions were used and the same native glycosides when enzyme inhibiting techniques were employed. The species investigated were *Digitalis mertonensis*, *Digitalis lanata*, *Digitalis orientalis*, and *Digitalis siberica*. It was also shown with the dried leaves of these species that 40 per cent methanol does not inhibit the enzyme that hydrolyzes the terminal glucose residue of the native glycosides. Furthermore, this alcohol concentration appeared to permit more complete hydrolysis as compared to the use of water or possibly 20 per cent methanol.

Although primary aqueous extracts of the fresh leaves of some of the digitalis species readily led to the isolation of some crystalline digitalis glycosides, the volume of water proved cumbersome, troublesome emulsions were encountered during extractions with organic solvents, and in some cases, there was incomplete extraction of the less water-soluble desglucoglycosides such as acetyl digitoxin and acetylgitoxin. The use of 15 per cent aqueous methanol was more effective than water and encouraged the use of greater concentrations of methanol or ethanol. Because 40 per cent concentrations of methanol did not inhibit enzymatic activity in the dried leaves of *Digitalis purpurea* whereas 66 per cent

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did, these concentrations were also used to extract the dried leaves of some of the other species of digitalis. In each case the results were analogous to those obtained with the dried leaves of *Digitalis purpurea*. The use of 40 per cent aqueous methanol permitted the ready extraction and subsequent detection of acetyl digitoxin in the case of *Digitalis lanata* whereas, when water was used to prepare the primary extract, the presence of acetyl digitoxin was difficult to establish.

The use of 40 per cent methanol to prepare the primary extracts had the following advantages: (*a*) more complete extraction of the less water-soluble desglucoglycosides, (*b*) much smaller volumes of solvent were needed, (*c*) easier filtration or percolation, and (*d*) less troublesome emulsions when the primary extract was extracted with organic solvents. The disadvantages that were encountered are: (*a*) incomplete extraction of the glycosides from the primary extract with organic solvents, (*b*) the necessity of diluting this primary extract with water or removal of part of the alcohol by distillation in order to obtain more complete extraction of the glycosides with organic solvents, and (*c*) considerably greater amounts of undesirable substances, some of which are colored, are obtained in this primary extract and in turn accompany the glycosides in the organic solvent extractions. The above discussion applied to both the qualitative identification and isolation studies.

In an attempt to improve upon the use of 40 per cent aqueous methanol, various concentrations of aqueous acetone or methyl ethyl ketone were tried. It was found that enzymatic activity in the dried leaves was not inhibited by 40 per cent aqueous acetone or 25 per cent aqueous methyl ethyl ketone. In these studies about the same advantages and disadvantages were encountered as with 40 per cent aqueous methanol. In the case of methyl ethyl ketone it was possible to add a sufficient amount of anhydrous sodium sulfate to effect a separation of the methyl ethyl ketone. A clean cut separation of the ketone was obtained and no emulsions were encountered. In this case the ketone served to facilitate the extraction of the glycosides in the primary extract and also served as the organic solvent to extract the glycosides from the primary extract. This methyl ethyl ketone extract contained considerable amounts of impurities in addition to the digitalis glycosides. Considerable amounts of impurities separated when a second solvent such as ether, isopropyl ether, etc., was added. The resultant mixed solvent solution of the glycosides was further partially depigmented by treatment with aqueous alkali as previously described (1).

Pigments, chiefly yellow in nature, accompany the organic solvent used to obtain the secondary extracts from the primary extracts. The quantities varied depending on the nature of the organic solvent or mixture of organic solvents. The solvents that have been tried are methyl isobutyl ketone, methylene dichloride plus ether (1:3), methyl ethyl ketone, and methyl ethyl ketone plus isopropyl ether (1:1). Considerable amounts of yellow pigment could be extracted from these secondary extracts with aqueous alkali and was most effective when the solvent was methylene dichloride ether (1:3). However, this procedure did not remove all the yellow pigments some of which were troublesome during qualitative paper chromatographic analyses. After removal of the organic solvent from the partially depigmented secondary extracts the residue was dissolved in methanol. In this solvent, especially if small amounts of water are added, more pigments can be removed with the base form of the ion exchange resin IRA-401.

Powdered dried leaves of *Digitalis mertonensis* were used in order to prepare larger quantities of digitoxin using 40 per cent methanol to prepare a primary extract. Contrary to previously reported findings (1), I was not able to obtain digitoxin as readily in a crystalline state, even though the final preparation contained a high percentage of digitalis glycosides. Analysis by paper chromatography, before and after deacetylation, revealed that this preparation contained considerable quantities of acetyl digitoxin together with larger amounts of digitoxin. These results were substantiated when the above experiment was repeated several times, on both small or large scales using dried leaves, and by a variety of extraction techniques. This was quite surprising since earlier techniques had led to the ready isolation of crystalline digitoxin when fresh leaves were employed and water or 15 per cent aqueous methanol was used as the solvent to prepare the primary extract. One might conclude that in the latter case the temperatures employed or enzymes in the fresh leaves led to a large amount of deacetylation of the acetyl digitoxin. If this be the case, it would differ from the results obtained with *Digitalis siberica* where only acetyl digitoxin could be detected even though, in this case, the primary extract was made with water or 15 per cent aqueous methanol in the same way. At the present stage of my investigations and for the lack of fresh leaves, no clear-cut conclusion can be drawn as to whether or not both purpurea glycoside A and lanatoside

A are present as native glycosides of *Digitalis mertonensis*. Further studies on fresh leaves should clarify this situation.

It should be pointed out at this time that *Digitalis mertonensis* is a hybrid of the *Digitalis purpurea* and *Digitalis grandiflora* species. The former yields purpurea glycoside A which, upon partial hydrolysis, leads to digitoxin whereas in the latter case lanatoside A is produced which can lead to acetyl digitoxin.

EXPERIMENTAL

The details of the paper chromatographic techniques used in these studies have been described previously (1–3). Both solvent systems I and II were used for the development of the paper chromatograms. The Raymond reagent or Jensen's trichloroacetic acid reagent (4) were used to detect the position of the glycosides on the paper. The extracts also were prepared as previously described (1, 2), unless otherwise specified.

Extraction With Methyl Ethyl Ketone.—Dried powdered *Digitalis siberica*, 200 Gm., was macerated with 1,000 cc. of 25% aqueous methyl ethyl ketone for eighteen hours. It was then packed in a percolator and slowly percolated with the same menstrum, and 1,500 cc. of percolate was collected. A sufficient amount of anhydrous sodium sulfate was added to effect a separation of the ketone (75% of the original amount of the ketone separated). The aqueous layer was extracted with a second quantity, 200 cc. of methyl ethyl ketone. The ketone extracts were combined and the solvent removed under vacuum. The residue was diluted with two heaping teaspoons of Celite filter aid and subsequently extracted with isopropyl ether—Skelly B, 1:1 (by volume) to remove some lipids and some pigments. It was then extracted with 100 cc. of methylene dichloride which was concentrated to a volume of 20 cc. and diluted with 60 cc. of ether. This extract was almost decolorized by extracting it with two 10-cc. portions of 5% aqueous potassium hydroxide. The organic solvents were removed and acetyl digitoxin crystallized from the residue using a mixture of methylene dichloride and isopropyl ether. The crystallization of acetyl digitoxin was very slow and required a considerable amount of time even when seeded.

A second method of utilization of methyl ethyl ketone was as follows: 10 Gm. of dried, powdered *Digitalis mertonensis* was heated at 60° with 150 cc. of water for one hour. The mixture was filtered and the drug washed with 50 cc. of hot (80°) water. The filtrate, 160 cc., was diluted with 40 cc. of methyl ethyl ketone. Anhydrous sodium sulfate then was added to effect a separation of the ketone. No emulsion was encountered. The aqueous layer was extracted with a second portion (30 cc.) of methyl ethyl ketone. The ketone extracts were combined and treated as described above. In addition, a methanolic solution (4 cc.) of the final product was treated with the powdered base form of IRA-401 which removed considerable additional amounts of yellow pigments. The final preparation, when examined paper chromatographically, revealed the presence of digitoxin and acetyl digitoxin. The identity of acetyl digitoxin was further established when, after deacetylation either by IRA-401 (base form) in hot 85% aqueous methanol or 0.1 *N* NaOH in methanol, paper chromatographic analyses showed the disappearance of acetyl digitoxin with an increase in the intensity of the digitoxin spot and the appearance of no new glycoside.

This experiment was designed to favor deacetylation because water and temperatures under 60° appear to favor deacetylation in the case of fresh leaves. In another experiment the primary aqueous methyl ethyl ketone extract was boiled for one hour; however, acetyl digitoxin could be readily detected. At the present stage of my researches the status of digitoxin and acetyl digitoxin as desglucoglycosides from *Digitalis mertonensis* needs further investigation.

Extraction With 40% Aqueous Methanol.—Dried, powdered *Digitalis mertonensis*, 10 Gm., was digested for five days with 100 cc. of 40% aqueous methanol. After filtration the marc was washed with 50 cc. of the same solvent. This primary extract was concentrated to remove most of the alcohol. It was then extracted with methyl isobutyl ketone and further purified in the usual way. Paper chromatographic examination showed the presence of digitoxin and acetyl digitoxin, and the latter seemed to predominate. This experiment was performed to see (*a*) if deacetylation would take place over this period of time and (*b*) to see if the glycosides would remain intact. Shorter periods of maceration gave similar results.

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Reactivity of Bisulfite With a Number of Pharmaceuticals*

By TAKERU HIGUCHI and LOUIS C. SCHROETER

Although bisulfite is presently widely employed as an antioxidant in preparations containing drugs which readily autoxidize; the antioxidant itself, according to the study described, undergoes reaction with a number of these compounds. Results of the present investigation show that sympathomimetic and other drugs which are *para*- or *ortho*-hydroxybenzyl alcohol derivatives react with bisulfite to yield corresponding sulfonic acid derivatives. Both amino and nitrobenzyl alcohols similarly conjugated (e. g., chloramphenicol) also appear to be subject to similar reactions. The resulting sulfonic acids seem to possess little or no activity.

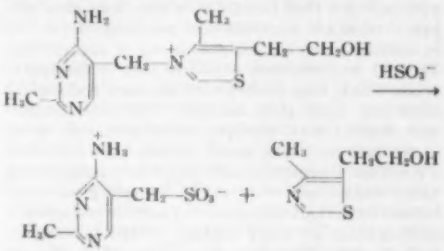
BISULFITE has been employed in pharmaceutical preparations as a stabilizer for many years. It has, in many cases, served very well in this capacity and inclusion of bisulfite into preparations as an antioxidant has become an accepted procedure. Attainment of a clear solution or elegant preparation through the use of bisulfite with easily oxidizable compounds has done much to support its use in formulations. In the present communication it is shown, however, that the stability of many important pharmaceuticals may be seriously impaired by the presence of bisulfite.

The reaction of aldehydes or certain ketones with bisulfite and the addition of bisulfite to the alkene linkage has been intensively studied (1, 2) and reactivity of these compounds is generally recognized. Reaction of alkyl halides with alkaline sulfite in aqueous solution to yield alkyl sulfonic acids is somewhat less known. Clutterbuck and Cohen (3) report the formation of a number of substituted benzyl sulfonic acids by boiling the corresponding benzyl bromide with aqueous sodium sulfite. The mechanism for this reaction appears to involve nucleophilic attack of sulfite at the partially positive carbon to yield a carbon-sulfur bond:

$$R-\overset{S^+}{CH_2}-\overset{S^-}{X} + SO_3^= \longrightarrow R-CH_2-SO_3^- + X^-$$

The interaction of aromatic nitro compounds with dilute sulfite solution to yield an arylaminosulfonic (arylsulfamic) acid and its sulfonation product takes place under similar conditions and is known as the Piria reaction (4). Nuclear sulfonation takes place *ortho* or *para* to the nitro group, presumably due to a nucleophilic attack at the partially positive carbon atom.

Early attempts to preserve rice polish extracts with sulfurous acid led to prompt and complete loss of thiamin activity. Further studies (5) showed that thiamin was cleaved by bisulfite into two compounds, one of which was a sulfonic acid. Thiamin cleavage of bisulfite has been demonstrated (6, 7) to occur as follows:



Experiments (6) with tritium indicated hydrogen is not exchanged in or out of the interannular methylene bridge of thiamin or of the sulfonic acid during the cleavage process. It appears that the pyrimidine sulfonic acid results from a nucleophilic attack on the partially positive methylene carbon.

In view of these observed reactions involving bisulfite, it is evident that the use of the antioxidant may result in instances of reduced stability. An example of this is the recently reported degradative route of epinephrine (8). The present study is concerned with this reaction and with determination of the types of drugs which may exhibit tendencies in this direction. Specifically, results of investigations on the reaction of bisulfite with epinephrine, ephedrine, 2-amino-1-phenylethanol, 2-methylamino-1-phenylethanol, catechol, resorcinol, quinhydrone, Methadren, N-acetylepinephrine, Synephrine, Neosynephrine, *p*-hydroxytoluene, *p*-methoxybenzyl alcohol, methyl-*p*-hydroxybenzoate, salicylamide, salicyl alcohol, *m*-hydroxybenzyl alcohol, *p*-hydroxybenzyl alcohol, *p*-aminobenzyl alcohol, *p*-aminobenzoic acid, 4-aminobenzoic acid, 4-aminosalicylic acid, tartaric acid, starch, chloramphenicol, and *p*-nitrobenzyl alcohol are reported.

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EXPERIMENTAL

Epinephrine solutions were prepared in 0.1 *M* acetate or phosphate buffers. The pH was determined at 25° using a Beckman model G pH meter. The solutions were flushed with nitrogen and stored under a positive nitrogen atmosphere until ready for use. These solutions served as blanks. Identical solutions were prepared containing, in addition, varying concentrations of sodium bisulfite or sodium sulfite. The pH of these solutions was adjusted at 25° to that of the appropriate blank and then flushed with nitrogen. Solutions were filled into a jacketed-polarimeter tube designed so that a nitrogen atmosphere was maintained above the solution at all times. Polarimetric measurements were made with a Zeiss-Winkel polarimeter using 589 mμ filtered sodium light at the temperature of the thermostat.

Bisulfite or sulfite solutions were prepared in 0.1 *M* acetate or phosphate buffers. The pH was determined at 25°. These blank solutions were flushed with nitrogen then rapidly filled into hard glass ampuls. Identical solutions were prepared containing, in addition, varying concentrations of epinephrine. The pH was adjusted at 25° to that of the appropriate blank, then flushed with nitrogen and rapidly filled into hard glass ampuls. The filled ampuls were flushed with nitrogen, evacuated, and sealed under vacuum. The sealed ampuls were stored at a constant thermostatically controlled temperature which varied less than 0.1°. Sampling was performed by periodically removing ampuls and quickly chilling in an ice-water mixture. Prior to analysis, ampuls were adjusted to 25°. The pH of all solutions subjected to elevated temperature was determined. Available bisulfite was determined by iodometric titration.

Reactivity of bisulfite with the following compounds was tested as described above: Ephedrine, U. S. P.; 2-amino-1-phenylethanol and 2-methylamino-1-phenylethanol synthesized from styrene bromohydrin according to the method of Read and Reid (9); catechol; resorcinol; quinhydrone; Methadren (N-methylepinephrine)[1]; N-acetylepinephrine, m. p. 135° obtained by hydrolysis (pH 7.5 phosphate buffer) of O³, O⁴, N-triacetylepinephrine; Synephrine and Neosynephrine[2]; *p*-hydroxytoluene; *p*-methoxy-benzyl alcohol; methyl-*p*-hydroxybenzoate; salicylamide; salicyl alcohol; *m*-hydroxybenzyl alcohol; *p*-hydroxybenzyl alcohol; *p*-aminobenzyl alcohol; *p*-aminobenzoic acid; 4-aminosalicylic acid; tartaric acid; and starch U. S. P.

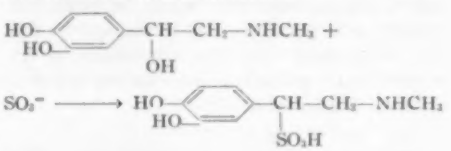
The reaction product resulting from bisulfite with *ortho*-hydroxybenzyl alcohol was prepared in quantity by heating in aqueous solution of 0.1 *M* salicyl alcohol and 0.2 *M* sodium sulfite in an evacuated, sealed ampul at 80° for periods up to one hundred hours. After cooling, the solution was extracted four times with equal volumes of ether. An aliquot was oxidized by alkaline permanganate and compared with *ortho*-sulfobenzoic acid obtained by acid hydrolysis of *saccharin*. The aqueous solution was made distinctly acid with hydrochloric acid (pH 2) and the solution again extracted with ether. An aliquot of the aqueous acid solution was boiled with 2,4-dinitrophenylhydrazine solution. Part of the aqueous acid solution was basified with sodium hydroxide and methylated under alkaline conditions with dimethylsulfate. The basic solution was extracted four times with equal volumes of ether. The methylated product was compared with known *ortho*-methoxybenzyl sulfonic acid synthesized by the method of Clutterbuck and Cohen (3) from the corresponding benzyl bromide and sodium sulfite solution. Both products were reacted with phosphorus pentachloride (0.5 of sod. sulfonate + 2 Gm. PCl_5) at 150° under reflux for forty minutes. Residue was extracted with benzene. Benzene solutions were evaporated to dryness. The residue was boiled with concentrated ammonium hydroxide solution.

Chloramphenicol and *p*-nitrobenzyl alcohol solutions were prepared in 0.1 *M* phosphate buffers and flushed with nitrogen prior to filling into ampuls. Chloramphenicol solutions were also prepared with L-threo-chloramphenicol in ethylene glycol-water (80:20). These solutions served as blanks. Identical solutions were prepared containing, in addition, varying concentrations of sodium bisulfite or sulfite. Ampuls were sealed under vacuum and stored for varying periods in a thermostat. Bisulfite loss from solutions was followed by iodometric titration. Chloramphenicol was assayed by a chromatographic procedure (10) and the optical activity (ethylene glycol-water solutions) determined at 25° in a 2-dm. micro polarimeter tube using 589 mμ light.

RESULTS AND DISCUSSION

Reaction with Epinephrine

Studies with epinephrine and optically active model compounds indicate that loss in optical activity occurs simultaneous to epinephrine and bisulfite loss. Evidence strongly suggests the asymmetric secondary alcohol carbon of epinephrine as the reactive site in this reaction:



The course of the reaction seems to be somewhat dependent on the pH of the reacting system.

At pH of 6.5, the reaction appears to be definitely a second-order reaction where $-d(\text{Ep})/dt = -d(\alpha)/dt = k\,(\text{Ep})\,(\text{Bi})$ as shown in Table I. The bisulfite concentration being in this instance the total of sulfite and bisulfite concentration. This relationship is also evident in the logarithmic type plot shown in Fig. 1 for systems starting with dissimilar concentrations as followed by bisulfite titration.

Loss of optical activity appears to occur at the same rate as loss of bisulfite and equilibrium optical activity of solutions is always zero. The following scheme will readily explain zero optical activity:

$$\text{Ep}(-) \xrightarrow[\text{(slow)}]{} \text{Ep}(\pm) \xrightarrow[\text{(fast)}]{(SO_3^=)} \text{EpBi}(\pm)$$

(± refers to sign of optical rotation)

[1] Samples generously supplied by Lakeside Laboratories, Milwaukee, Wis.

[2] Samples generously supplied by Sterling-Winthrop Research Institute, Rensselaer, N. Y.

TABLE I.—INITIAL RATES OF BISULFITE AND OPTICAL ACTIVITY LOSS FROM SOLUTIONS BUFFERED AT pH 6.5 WITH 0.1 *M* PHOSPHATE[a]

Temp., °C.	Soln. A[b] $-da/dt$ (mole l^{-1} sec.$^{-1}$)	Soln. B[c] $-d(\mathrm{Bi})/dt$ (mole l^{-1} sec.$^{-1}$)
90	6.63×10^{-6}	6.73×10^{-6}
84	3.65×10^{-6}	3.58×10^{-6}
78	2.09×10^{-6}	1.96×10^{-6}
72	1.21×10^{-6}	1.09×10^{-6}
66	7.12×10^{-7}	5.96×10^{-7}

[a] Initial rate of loss for reactant present in lesser concentration where concentration change is less than 10%.
[b] Solution A—$2.75 \times 10^{-3} M$ epinephrine, $9.60 \times 10^{-3} M$ sodium bisulfite. Optical activity assumed to be due to *levo*-epinephrine.
[c] Solution B—$1.92 \times 10^{-3} M$ sodium bisulfite, $5.50 \times 10^{-3} M$ epinephrine.

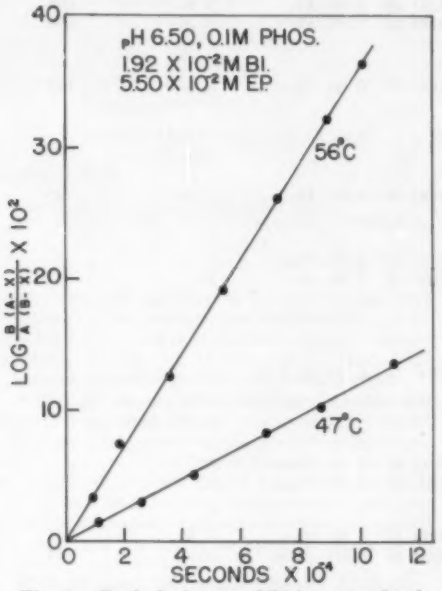


Fig. 1.—Typical plots establishing second-order characteristics of epinephrine (Ep)-sodium bisulfite (Bi) reaction at pH 6.50 and various temperatures. A—5.50×10^{-3} *M* epinephrine base (Ep) and B—1.92×10^{-3} sodium bisulfite (Bi). Rate constants were calculated from slopes: $k = m \times 2.303/A - B$.

This is the form of the Sn_2 mechanism. Implied in this mechanism is dependence of reaction only on epinephrine. If this were the only mechanism, the rate of bisulfite loss would equal the rate of loss of optical activity; however, observed second-order kinetics at higher pH values suggest additional pathway(s). Prior studies (8) indicate that this mechanism may make significant contributions to the reaction at pH values below 4.5.

At higher pH values, a slow, rate-determining Sn_2 reaction between epinephrine and bisulfite followed by rapid racemization seems a distinct possibility. In this case, one must assume rapid racemization since the Sn_2 mechanism generally involves inversion of configuration at the optical center and this would tend to create a positive equilibrium optical rotation. Kinetic evidence indicating that the reaction is second order at pH 6.5 suggests the following:

$$\mathrm{Ep}(-) + \mathrm{Bi} \xrightarrow[\text{slow}]{} \mathrm{EpBi}(+) \underset{\text{(fast)}}{\rightleftharpoons} \mathrm{EpBi}(-)$$

Isolation of bisulfite-salicyl alcohol as discussed below strongly suggests the formation of a benzyl sulfonic acid and, if the reaction in this model compound is similar to that of epinephrine, one would expect that the epinephrine-bisulfite product is also a benzyl sulfonic acid.

Bisulfite Reactivities of Other Compounds

Results of a screening test with a number of pharmaceuticals having close or distant similarity to epinephrine are given in Table II. In all cases a substantial excess of the drug was used and the bisulfite concentration followed by iodometric titration. The rate is expressed in terms of pseudo half-life at the given concentrations of the medicinal agents.

Ephedrine and Analogs.—Variation of reactant molarity, pH, or buffer failed to give a detectable reaction between ephedrine and bisulfite. Structural differences between ephedrine and epinephrine suggested that either the phenolic hydroxyls of epinephrine or the slightly different side chain accounted for the lack of reactivity of ephedrine with bisulfite. Failure of both 2-amino-1-phenylethanol and 2-methylamino-1-phenylethanol to react with bisulfite indicated side chain involvement to be a questionable hypothesis.

Dihydroxy Benzenes.—The isomeric dihydroxybenzenes (catechol, resorcinol, and the hydroquinone-quinone combination) also failed to react with bisulfite under standard conditions of the study. Reaction between bisulfite and hydroquinone and resorcinol on prolonged heating has been reported (11) but no loss of bisulfite was detected in our study. This seemed to indicate that the overall reaction between epinephrine and bisulfite involved the phenolic function with at least part of the side chain.

Epinephrine Analogs.—Neosynephrine, differing from epinephrine only in lacking the 4-hydroxyl, failed to react with bisulfite; however, Synephrine (*p*-methylaminoethanol phenol) reacted rapidly with bisulfite. Methadren (N-methylepinephrine) likewise underwent rapid reaction with bisulfite further indicating that the *p*-hydroxybenzyl alcohol portion of the molecule was involved in the reaction. Under identical reaction conditions, the pseudo first-order rate constant for the Methadren-bisulfite reaction was found to be approximately the same as that for the epinephrine-bisulfite reaction. Basicity difference between the secondary and tertiary amine apparently does not much influence the reaction rate. The N-acetylepinephrine reaction with bisulfite occurs at a slower rate than that found with epinephrine or Synephrine. The protonated amine in epinephrine, pKa 8.55, and Synephrine, pKa 8.62 (12), appears to facilitate the reaction.

Catechol-Phenylephrine Combination.—Evidence against sequential reaction of bisulfite with side chain, followed by attack on phenolic hydroxyls

TABLE II.—REACTIVITY OF VARIOUS COMPOUNDS AT 68° WITH 1.92×10^{-2} *M* SODIUM BISULFITE[a]

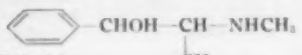
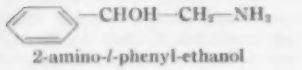
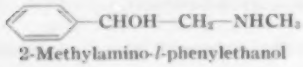
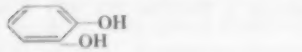
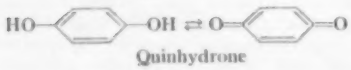
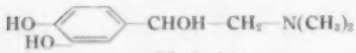
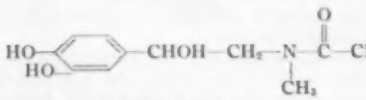
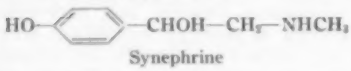
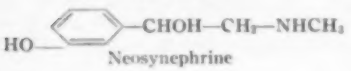
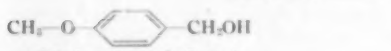
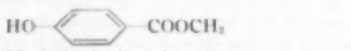
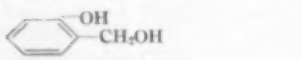
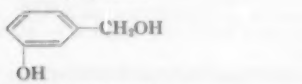
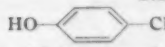
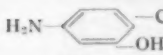
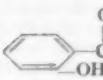
Compound	Concn.	Buffer pH[b]	Bisulfite Half-Life, hr.	Total Residence Time, hr.
 Ephedrine	0.023 *M*	5.50, Ac.	N.R.	76
	0.115 *M*	5.50, Ac.	N.R.	76
	0.023 *M*	8.50, Phos.	N.R.	67
 2-amino-*l*-phenyl-ethanol	0.073 *M*	5.50, Phos.	N.R.	144
 2-Methylamino-*l*-phenylethanol	0.033 *M*	5.00, Ac.	N.R.	117
 Catechol	0.090 *M*	4.50, Ac.	N.R.	168
	0.090 *M*	6.70, Phos.	N.R.	143
Resorcinol	0.090 *M*	6.80, Phos.	N.R.	144
 Quinhydrone	0.090 *M*	6.50, Phos.	N.R.	96
 Methadren	0.043 *M*	5.50, Phos.	30	...
	0.026 *M*	5.50, Ac.	42	...
 N-Acetylepinephrine	0.016 *M*	6.50, Phos.	83	...
 Synephrine	0.050 *M*	6.40, Phos	32	...
	0.055 *M*	6.98, Phos.	10	...
 Neosynephrine	0.050 *M*	5.50, Phos.	N.R.	144
	0.050 *M*	6.70, Phos.	N.R.	144
Catechol	0.045 *M*			
Phenylephrine	0.025 *M*	5.50, Ac.	N.R.	110
p-Hydroxytoluene	0.092 *M*	6.50, Phos.	N.R.	168
 *p*-Methoxybenzyl alcohol	0.040 *M*	8.00, Phos.	N.R.	96
 Methyl-*p*-hydroxybenzoate	0.033 *M*	7.00, Phos.	N.R.	120
 Salicyl alcohol	0.080 *M*	6.50, Phos.	10	...
 *m*-Hydroxybenzyl alcohol	0.040 *M*	6.70, Phos.	N.R	48
	0.080 *M*	6.70, Phos.	N.R.	120

TABLE II—*Continued*

Compound	Concn.	Buffer pH[b]	Bisulfite Half-Life, hr.	Total Residence Time, hr.
 *p*-Hydroxybenzyl alcohol	0.040 *M*	6.50, Phos.	9	...
p-Aminobenzyl alcohol	0.080 *M*	7.20, Phos.	11	...
p-Aminobenzoic acid	0.036 *M*	6.80, Phos.	N.R.	96
 4-Aminosalicylic acid	0.033 *M*	6.80, Phos.	N.R.	96
 Salicylamide	0.036 *M*	7.00, Phos.	N.R.	120
HOOC—CHOH—CHOH—COOH Tartaric acid	0.067 *M*	7.20, Phos.	N.R.	120
Starch U. S. P.	0.5%	7.20, Phos.	N.R.	120

[a] Solutions stored in evacuated, sealed ampuls.
[b] 0.1 *M* acetate (Ac.) or 0.1 *M* phosphate (Phos.) buffers.

or vice versa was provided by the study of the catecol-phenylephrine combination with bisulfite. Failure of the combination to react with bisulfite further substantiates the idea of the *p*-hydroxybenzyl alcohol moiety of epinephrine as the active portion.

***p*-Hydroxytoluene, *p*-Methoxybenzyl Alcohol, and Methyl-*p*-hydroxybenzoate.**—The simple model compounds, *p*-hydroxytoluene, *p*-methoxybenzyl alcohol, and methyl-*p*-hydroxybenzoate, show no reaction with bisulfite under standard conditions. These compounds are all *para*-substituted benzenes; however, resonance contributions to give the quinoid structure are not nearly so large as in the *para*-hydroxybenzyl alcohol.

Hydroxybenzyl and *p*-Aminobenzyl Alcohols.—The isomeric hydroxybenzyl alcohols show very significant information in that the *ortho* and *para* isomers react rapidly with bisulfite while the *meta* does not. This confirms the results obtained with Synephrine and Neosynephrine which are *para*- and *meta*-hydroxybenzyl alcohol derivatives, respectively. Involvement of contributing resonance forms seems apparent at this point and studies with *p*-aminobenzyl alcohol strongly suggest the *para*-quinoid structure of a benzyl alcohol as the reactive form. Further, the reaction of *p*-amino-benzyl alcohol obviates consideration of the phenolic group *per se*.

***p*-Aminobenzoic Acid, 4-Aminosalicylic Acid, and Salicylamide.**—Studies with *p*-aminobenzoic acid, 4-amino salicylic acid, and salicylamide indicate that the aromatic amino group and the phenolic hydroxyl group do not account for the bisulfite reaction in themselves. These groups, when *para* or *ortho* to the benzyl alcohol, make the quinoid resonance form a distinct possibility and activate the alcohol.

Tartaric Acid and Starch U. S. P.—As expected, no reaction was detected with the common formulation ingredients, tartaric acid, and starch U. S. P.

Salicyl Alcohol–Bisulfite Product.—Reaction of salicyl alcohol with sodium bisulfite in a sealed tube yields a polar compound insoluble in ether and showing the same ultraviolet spectrum as salicyl alcohol. Combustion analysis of the product indicated the formula: $C_7H_7O_4NaS$. Alkaline permanganate oxidation of the product yields no isolable or ultraviolet-detectable aromatic products. If the reaction involved substitution at the phenolic hydroxyl group, oxidation should have yielded *ortho* sulfobenzoic acid.

Acidification of the salicyl alcohol-bisulfite product failed to yield an ether-soluble product and boiling the acid solution with 2,4-dinitrophenylhydrazine failed to yield a derivative. An aldehyde intermediate in the reaction seems unlikely. Methylation of the salicyl alcohol-bisulfite product with dimethylsulfate under basic conditions produced a polar compound insoluble in ether which showed a doublet peak in the ultraviolet. The doublet peak at 273 and 280 mμ was identical with the *ortho*-methoxybenzyl chromophore.

The methylated product and known *ortho*-methoxybenzyl sulfonic acid were subjected to alkaline permanganate oxidation under a reflux condenser for periods up to six hours. Less than 10% *ortho*-methoxybenzoic acid was isolated from the mixture. The remainder of the product in each case is unchanged indicating the inordinate resistance of benzyl sulfonic acids to alkaline hydrolysis and alkaline oxidation.

Both the methylated product and known *ortho*-methoxybenzyl sulfonic acid failed to yield sulfuryl chloride derivatives by usual phosphorus pentachloride procedure. Reaction residues contained varying amounts of *ortho*-methoxybenzyl chloride.

Formation of benzyl chlorides rather than expected sulfonyl chlorides has been previously reported (1). Apparently strict control of reaction conditions is necessary to obtain benzyl sulfonamides since Clutterbuck and Cohen (3) report preparations of sulfonamides derivatives for substituted benzyl sulfonic acids other than *ortho*- and *para*-methoxybenzyl sulfonic acid.

Chloramphenicol.—Degradation of chloramphenicol induced by bisulfite appears to be considerably more complex than that found with epinephrine. Certain similarities may be seen in Fig. 2 in which both the log of chloramphenicol loss as determined by chromatography assay and log of optical activity are plotted against time. The reaction follows pseudo first-order kinetics since the bisulfite is initially in excess. Loss in optical activity occurs at a rate much greater than that observed in the absence of bisulfite. Presence of two optically active carbon atoms imposes an element of uncertainty in evaluating polarimetric data and, to be sure, the initially levorotatory solutions were found to yield dextrorotatory solutions at equilibrium (15 half-lives). Further, the stoichiometry of the reaction is complicated by the reductive tendency of bisulfite with nitro compounds and its nucleophilic reactivity toward alkyl halides.

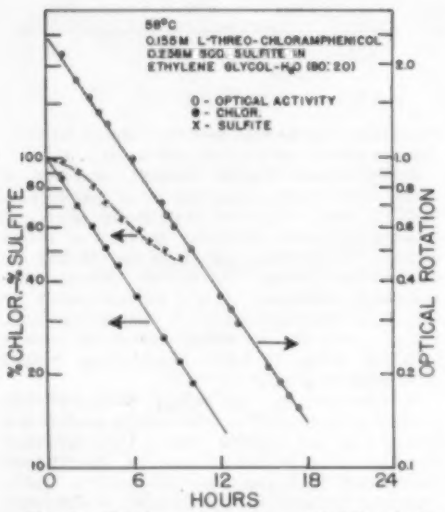


Fig. 2.—Ethylene glycol-water (80:20) solution of 0.155 *M* L-threo-chloramphenicol and 0.238 *M* sodium sulfite at 58°. Per cent chloramphenicol (Chlor.) and per cent sulfite plotted logarithmically on left axis; optical rotation in degrees measured at 25° in 2-dm. polarimeter tube plotted logarithmically on right axis.

Studies with the model compound, *p*-nitrobenzyl alcohol, indicated very rapid loss of bisulfite from solutions with concomitant appearance of a polar compound. Production of the polar product was followed by partition studies of the solutions. As with chloramphenicol, the initial rate loss of the nitro compound is near that of bisulfite; however, as the reaction proceeds, the bisulfite loss tends to increase, indicative of side reactions.

GENERAL OBSERVATIONS

Comparative study of epinephrine and ephedrine analogs and model compounds clearly indicated that the reaction between epinephrine and bisulfite is not unique. The basis for the reactivity of epinephrine or similar drugs with bisulfite under conditions of the experiment has been shown to involve the hydroxybenzyl alcohol portion of the molecule.

The influence of position in the reactivity of the isomeric hydroxybenzyl alcohols indicated that resonance contributions to a quinoid structure would account for lack or reactivity of *meta* isomer. Correctness of this concept was substantiated in studies with *p*-aminobenzyl alcohol, which has considerable quinoid resonance contribution.

The chloramphenicol study showed certain similarities to the epinephrine reaction in that optical activity loss occurs at nearly the same rate as the loss of chloramphenicol. The reaction appears to be more complex than that observed with hydroxybenzyl alcohol derivatives. Studies with the model compound *p*-nitrobenzyl alcohol indicated strong possibility for nitro reduction.

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(1) Suter, C. M., "The Organic Chemistry of Sulfur," John Wiley & Sons, New York, N. Y., 1944, pp. 126, 98, 101.
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(3) Clutterbuck, P. W., and Cohen, J. B., *J. Chem. Soc.*, **123**, 2507(1923).
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Protective Effects of Air Under Pressure on Certain Pharmaceuticals During Steam Autoclaving*

By LEON LACHMAN, DONALD JACONIA, and PHILIP EISMAN

Pharmaceutical products such as jellies packaged in collapsible tubes cannot be properly sterilized in conventional steam autoclaves because of expansion or bursting of the tubes. Similarly, leakage at the cap occurs during the sterilization of screw-capped bottles. In this report, a redesigned steam autoclave is described which permits the practical sterilization of these pharmaceutical formulations. This autoclave is a dual-control unit permitting the introduction of air under pressure into the chamber so as to obtain a total air plus steam pressure greater than that of the vapor pressure within the containers. In addition, this unit allows for a reduction in chamber temperature without loss of chamber pressure during the cooling cycle. The suitability of this dual-control autoclave for the sterilization of the aforementioned products under various conditions of temperature and pressure was determined. Data are presented indicating that these pharmaceutical products can be effectively sterilized without deleterious effects to the containers. The jellies and solutions used in this investigation were heavily contaminated with *B. cereus* spores. Recordings of the temperature within the containers and chamber show that there is a lag in the rise and fall of the temperature within the containers as compared to that of the chamber.

ALTHOUGH there are a number of techniques involving heat which may be employed for the sterilization of various pharmaceutical dosage forms, there is little doubt that the most dependable procedure is the one which utilizes steam under pressure. Unless the product to be sterilized is deleteriously affected by the temperature and pressure involved, the autoclave serves admirably in destroying all forms of microbial life.

The effectiveness of this method of sterilization is dependent upon two essential factors, namely moisture and heat. It is a universally accepted fact that the thermal destruction of microorganisms parallels the principles involved in the heat coagulation of proteins. In the presence of adequate amounts of water, the coagulation of proteins occurs at temperatures well below that necessary to achieve the same results when the moisture content is appreciably reduced. This relationship between coagulation temperature of proteins and moisture content was presented in a classic paper by Lewith (1) in 1890, a summary of which is shown in Table I.

TABLE I.—COAGULATION OF EGG ALBUMIN WITH VARYING MOISTURE CONTENT

Egg Albumin-Water Mixtures	Temperature of Coagulation in 30 Min., ° C.
Aqueous solution	56
With 25% water	74–80
With 18% water	80–90
With 6% water	145
Water free	160–175

The term autoclave, as it is usually applied, refers to a chamber capable of accepting pressurized steam under controlled conditions. The sterilizing power of the steam is a function of its temperature and not its pressure. However, since the temperature in the autoclave is proportional to its steam pressure, an increase in pressure results in an increase in temperature.

Not all pharmaceutical products can be suitably sterilized in an autoclave. In the process of autoclaving, pressure is built up within the container being sterilized because of the vapor pressure of water and the partial pressures of volatile components, if present in the formulation. Under these circumstances, the internal pressure could be substantially greater than the steam pressure within the chamber itself. This pressure differential could result in collapsible tubes expanding and bursting at the crimp end, screw-capped bottles losing a portion of their contents, and the breaking of glass containers. Furthermore, during the cooling phase of the sterilization process, the chamber almost always cools at a faster rate than does the contents of the sterilized packages. As a consequence, pressures within these containers are substantially higher than that of the chamber, and unless these containers are properly sealed and adequately constructed, deleterious effects may result. Containers such as collapsible tubes, whether constructed of aluminum or plastic, bottle assemblies fitted with screw caps, and Penwall bottles with their not-too-firmly anchored caps are particularly subject

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to this undesirable effect of uneven pressures. For this reason, such products as sterile jellies and ophthalmic solutions are often commercially prepared according to aseptic techniques.

This difficulty, namely the bursting of collapsible tubes and the leakage of solution from screw-capped containers, can be overcome by the simple expediency of controlling chamber pressure through the introduction into the chamber of air under pressure during the heating and subsequent cooling cycles. However, the addition of air to steam results in a mixture which, at any particular total pressure, has a temperature lower than that obtained with saturated steam alone at the same pressure (2). This relationship of air plus steam pressure to temperature is exemplified by the data presented in Table II. Thus, if one operates an autoclave in which only one-half of the air in the chamber has been removed, a pressure of 15 p. s. i. results in a temperature of 112° as contrasted to a temperature of 121° in the case where all the air has been evacuated. As illustrated in Fig. 1, one can predetermine the temperature resulting from the use of any proportion of air to steam by plotting the chamber temperatures against different proportions of air and steam at a constant total pressure.

Consideration was then given to the possibilities of utilizing air-steam mixtures in the sterilization of products not normally amenable to conventional autoclaving treatment because of leakage or other undesirable physical effects to the containers. Excellent examples of such products are pharmaceutical jellies packaged in collapsible aluminum tubes and ophthalmic solutions in screw-capped bottles. Through the cooperation of the Wilmot Castle Company Laboratories in Rochester, N. Y., an autoclave was redesigned so as to permit the regulation of chamber temperature and pressure independently of each other. This redesigned autoclave was made available to us for use in this study and hereafter will be referred to as the "dual-control autoclave."

The objects of this study were (*a*) to determine the sterilizing effectiveness of contaminated pharmaceutical jellies and ophthalmic solutions subjected to various combinations of temperature and pressure in the dual-control autoclave; and (*b*) to determine the ability of the tubes and bottles to withstand physical changes under these conditions. In addition, simultaneous temperature recordings were made of the autoclave chamber and the contents of the containers in order to determine whether a substantial time lag occurs between the rise and fall of the temperature within the tubes and bottles in relation to the chamber temperature.

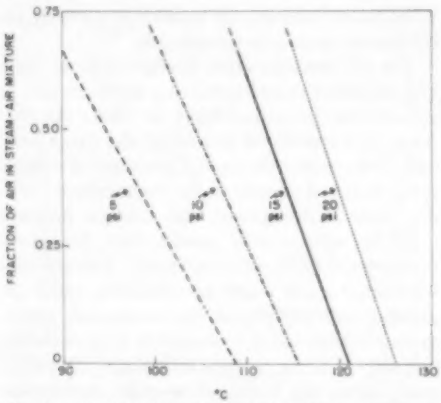

Fig. 1.—A plot of air-stream mixtures *vs.* temperature at different total gauge pressure.

EXPERIMENTAL

Design of the Dual-Control Autoclave

As shown in Fig. 2, this unit is essentially an adapted steam autoclave of the ordinary type in which it is possible (*a*) to build up pressure without heat, (*b*) to utilize temperatures below 100° without pressure, and (*c*) to apply varying temperatures and pressures concurrently. These features are brought about by the introduction of solenoid-controlled air and steam at the inlet port of the sterilizer. The steam serves to heat the chamber and when the desired temperature is reached, air is introduced to bring the chamber to the required pressure. For example, in order to operate the autoclave at 105° at 15 p. s. i., steam is passed into the chamber until a temperature of 105° is reached. Since at this temperature there is a pressure of 3 p. s. i., air is admitted until the total pressure of 15 p. s. i. is obtained. The thermo and pressure switches on the chamber automatically maintain the desired temperature and pressure during the sterilizing cycle. A pulsator system

TABLE II.—STERILIZER TEMPERATURE WITH VARIOUS DEGREES OF AIR DISCHARGE

Gauge Pressure, p. s. i.	Saturated Steam Complete Air Discharge, °C.	Two-Thirds Air Discharge, °C.	One-Half Air Discharge, °C.	One-Third Air Discharge, °C.	No Air Discharge, °C.
5	109	100	94	90	72
10	115	109	105	100	90
15	121	115	112	109	100
20	126	121	118	115	109
25	130	126	124	121	115
30	135	130	128	126	121

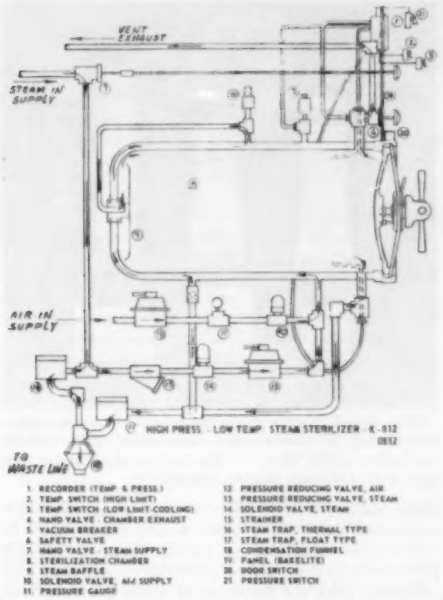
VENT EXHAUST
STEAM IN SUPPLY
AIR IN SUPPLY
TO WASTE LINE
HIGH PRESS. - LOW TEMP. STEAM STERILIZER - K-032
1. RECORDER (TEMP. & PRESS.)
2. TEMP. SWITCH (HIGH LIMIT)
3. TEMP. SWITCH (LOW LIMIT-COOLING)
4. HAND VALVE - CHAMBER EXHAUST
5. VACUUM BREAKER
6. SAFETY VALVE
7. HAND VALVE - STEAM SUPPLY
8. STERILIZATION CHAMBER
9. STEAM BAFFLE
10. SOLENOID VALVE, AIR SUPPLY
11. PRESSURE GAUGE
12. PRESSURE REDUCING VALVE, AIR
13. PRESSURE REDUCING VALVE, STEAM
14. SOLENOID VALVE, STEAM
15. STRAINER
16. STEAM TRAP, THERMAL TYPE
17. STEAM TRAP, FLOAT TYPE
18. CONDENSATION FUNNEL
19. PANEL (BAKELITE)
20. DOOR SWITCH
21. PRESSURE SWITCH

Fig. 2.—A comprehensive description of the dual-control autoclave.

adjusts the flow of steam and compressed air into the chamber upon demand, utilizing steam at 132° and air at 32°. By this device prolonged surges of steam and air, which would influence temperature and pressure stability, are thereby prevented. The dual-control autoclave is capable of operation within a range of 0–30 p. s. i. and 50–127°.

The autoclave is equipped with a timer which controls the duration of the sterilizing cycle once the desired temperature of the container contents has been reached. At the point when the chamber temperature has reached the autoclaving temperature, sufficient air under pressure is automatically admitted to obtain the predetermined total pressure. At the end of the sterilization cycle, the timer permits the autoclave to gradually exhaust steam at a rate which can be preset and calls for ambient air which enters the chamber through a sterile filter, thereby preventing a drop in chamber pressure during the cooling cycle. However, the cooling cycle can be terminated at will by manually opening the exhaust valve. The chamber temperature and pressure as well as the temperature of the contents in the containers are constantly recorded during the sterilization cycle.

Materials

The jelly used in this study was N, N-dimethyl-N′-benzyl-N′ (alpha-pyridyl) ethylenediamine hydrochloride (Pyribenzamine anesthetic jelly 2%) containing 0.5% chlorobutanol as preservative and formulas of this jelly with the following modifications: (*a*) minus chlorobutanol, (*b*) minus Pyribenzamine HCl, (*c*) minus both chlorobutanol and Pyribenzamine HCl.

The ophthalmic solutions used were 2-(4-*tert*-butyl-2,6-dimethyl) benzyl-2-imidazoline hydrochloride (Otrivin 0.05%) containing 1:100,000 phenylmercuric acetate as preservative, as well as the same solution minus phenylmercuric acetate and, as a control, 0.9% sodium chloride solution.

B. cereus spores.

Collapsible aluminum tubes.

Amber, screw-capped, 15-ml. bottles with heat resistant screw caps made of black phenolic mineral composition.

Procedure

Sterilization in Dual-Control Autoclave.—A number of filled tubes of jelly and bottles of ophthalmic solutions were subjected to several temperatures for periods of time as indicated in Tables III and IV. The pressure within the chamber as well as the temperatures of the chamber and container contents were recorded throughout the sterilization cycle. For comparative purposes, other filled containers were treated at varying temperatures in a conventional autoclave for thirty-minute periods.

Microbiological Testing.—The ophthalmic solutions and jellies to be sterilized in the dual-control autoclave were contaminated with a concentrated saline suspension of *B. cereus* spores prior to filling into the bottles and collapsible aluminum tubes, respectively. Thorough mixing was achieved by means of a Cenco electric stirrer, as evidenced by replicate platings. After subjecting the tubes and bottles to the conditions of autoclaving as described in Tables III and IV, plate counts for survivors and sterility tests in fluid thioglycollate medium were performed.

TABLE III.—STERILIZING CONDITIONS FOR PHARMACEUTICAL JELLIES IN COLLAPSIBLE TUBES

Autoclave	Temperature, °C.	Gauge Pressure, p. s. i.	Duration, min.
Dual control	95	10	30
	95	15	30 and 45
	105	10	30
	105	15	30 and 45
	105	20	30
	105	25	30
	105	30	30
	115	10	30
	115	15	30 and 45
	115	20	30
	115	25	30
	115	30	30
Conventional	100	..	30
	105	3	30
	115	10	30

TABLE IV.—STERILIZING CONDITIONS FOR OPHTHALMIC SOLUTIONS IN SCREW-CAPPED BOTTLES

Autoclave	Temperature, °C.	Gauge Pressure, p. s. i.	Duration, min.
Dual control	95	10	30
	95	15	30
	105	15	30
	105	20	30
	105	25	30
	115	15	30
	115	25	30
	115	30	30
Conventional	100	..	30
	105	3	30
	115	10	30

Pyribenzamine Anesthetic Jelly 2%.—The contents of each tube were placed into a sterile beaker and mixed thoroughly. Duplicate 1.0-Gm. portions of jelly were diluted in trypticase soy broth, plated on trypticase soy agar, and survivors determined. In addition, duplicate 1.0-Gm. portions of material were placed into 200-ml. amounts of fluid thioglycollate medium and incubated at 37° for fourteen days. The absence of growth indicated sterility.

Otrivin Ophthalmic Solution 0.05%.—Duplicate 1.0-ml. portions of solution were diluted in broth and then plated in trypticase soy agar containing 0.03% thioglycollic acid (thioglycollic acid was used to neutralize possible carry-over of phenylmercuric acetate) and survivors determined. Additional 1.0-ml. portions of solution were transferred to 200-ml. amounts of fluid thioglycollate medium and the bottles incubated for fourteen days at 37°. The absence of growth at the end of this period of time indicated sterility.

RESULTS AND DISCUSSION

Antibursting and Antileaking Effect of Air Pressure During Steam Autoclaving.—Since the water content in the jelly is approximately 85% and the ophthalmic preparation is an aqueous solution, the pressure in the tubes and bottles at the varying autoclaving temperatures can be essentially attributed to the vapor pressure of water at these respective temperatures. The effect of the components in the formula upon the pressure within the containers is insignificant since they are present in negligible quantities as compared to water. Since the vapor pressure of a liquid is a constant quantity at any temperature and is independent of the amount of liquid present (3), it is possible to obtain the vapor pressure of water from the critical tables.

Through the utilization of the dual-control autoclave, it is possible to prevent swelling or the bursting of collapsible tubes of pharmaceutical jellies as well as the leakage of solution from screw-capped bottles by the adjustment of the pressure within the chamber to approximate that of the contents within the containers.

The physical condition of the filled tubes of jelly was found essentially unaltered when treated in the dual-control autoclave under the conditions outlined in Table III. This absence of deleterious effect is also shown in Fig. 3. Careful inspection of these jelly-filled tubes treated below 20 p. s. i. at 105° and below 25 p. s. i. at 115° showed barely perceptible expansion. However, at pressures of 20 p. s. i. and above at 105° and at a pressure of 25 p. s. i. and above at 115° no evidence of any deleterious effects could be noted.

The glass containers as well as the screw caps used for the ophthalmic solution bore no visible evidence of change upon sterilization in the dual-control autoclave at any of the autoclaving conditions noted in Table IV. As already indicated, a special heat resistant cap of black phenolic mineral composition was used in this study.

Figure 3 also illustrates the undesirable effects that occur within the conventional autoclave in which the chamber pressure is strictly a function of steam alone. From a physical standpoint, the effect on the tubes has been disastrous.

Microbiological Studies.—The products used in these studies were Pyribenzamine anesthetic jelly preserved with 0.5% chlorobutanol and Otrivin ophthalmic solution preserved with 1:100,000 phenylmercuric acetate. Previous experiences had indicated that the presence of these preservatives renders it possible to sterilize these products at temperatures substantially below 121°. Since the dual-control autoclave permits the treatment of filled collapsible tubes and screw-capped bottles to a variety of temperatures without deleterious effects to the containers, experiments were performed in which these products were heated in this autoclave at varying temperatures and pressures.

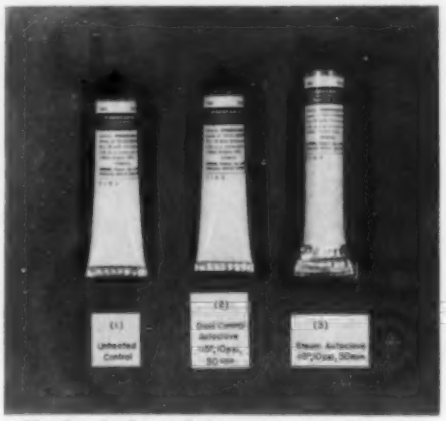

Fig. 3.—A photo of the appearance of the tubes containing Pyribenzamine anesthetic jelly before and after sterilization in the dual-control autoclave and conventional steam autoclave.

In one experiment a quantity of jelly was contaminated with approximately 180,000 *B. cereus* spores per Gm. of material. The contaminated jelly was filled into one-ounce collapsible tubes and crimped. At the Wilmot Castle Laboratories, the tubes were treated in the dual-control autoclave under varying conditions of temperature and pressure and then returned to our laboratories for sterility tests and survivor counts. The results of this experiment are shown in Table V and indicate that the product is effectively sterilized at a temperature as low as 95°.

TABLE V.—INFLUENCE OF TEMPERATURE, PRESSURE (AIR PLUS STEAM), AND DURATION ON THE STERILIZATION OF PYRIBENZAMINE ANESTHETIC JELLY

Run No.	No. of Tubes Tested	Temperature, °C.	Pressure, p. s. i.	Time, min.	Results
1	3	95	10	30	Sterile
2	3	95	15	30	Sterile
3	3	95	15	45	Sterile
4	3	105	10	30	Sterile
5	3	105	15	30	Sterile
6	5	105	20	30	Sterile
7	5	105	25	30	Sterile
8	5	105	30	30	Sterile
9	3	115	10	30	Sterile
10	3	115	15	30	Sterile
11	5	115	20	30	Sterile
12	5	115	25	30	Sterile
13	5	115	30	30	Sterile

Unheated control—180,000 organisms/Gm. Inoculum—300,000 organisms/Gm.

It was also felt desirable to subject to these same conditions tubes of jelly devoid of either chlorobutanol, or Pyribenzamine HCl, or both, contained in collapsible aluminum tubes. According to experiments conducted in our laboratories earlier, Pyribenzamine HCl is capable of exerting a mild but measurable preservative action and it was, therefore, desirous to establish the role this substance plays in the sterilization, at marginal temperatures, of *B. cereus* contaminated jelly. In addition, it was also desirable to determine the effect of the presence of chlorobutanol on the effectiveness of submarginal temperatures in destroying microorganisms in Pyribenzamine anesthetic jelly. Accordingly, four different batches of jelly were prepared. They consisted of (*a*) the complete formula, (*b*) the product minus the chlorobutanol, (*c*) the product minus Pyribenzamine, and (*d*) the product minus both chlorobutanol and Pyribenzamine. The results of this experiment to determine the influence of Pyribenzamine and chlorobutanol on the sterilizing capabilities of the autoclave at 95° and 105° are shown in Table VI.

From these results it is evident that, whereas the complete formula can be sterilized at a temperature of 95°, the removal of either chlorobutanol or Pyribenzamine HCl renders it impossible to sterilize the jelly at this temperature. It is only at 105° that these formulations can be effectively sterilized. Thus chlorobutanol and Pyribenzamine play important roles in the sterilization of this preparation at temperatures below 105°.

Similar experiments were performed with preserved (phenylmercuric acetate 1:100,000) and unpreserved Otrivin ophthalmic solutions. In addition, and for reference purposes, a solution of 0.9% sodium chloride was also tested.

According to data presented in Table VII, screw-capped bottles of Otrivin ophthalmic solutions, unpreserved or preserved with 1:100,000 phenylmercuric acetate, as well as 0.9% sodium chloride solution were sterilized within thirty minutes at temperatures of 95° and above and at pressures of 10 p. s. i. and greater.

Temperature Relationship Between Tube and Bottle Contents and Autoclave Chamber.—During the various autoclaving experiments, the temperature differential throughout the sterilization and subsequent cooling cycles of the chamber and the material being treated was recorded. This was accomplished through the use of thermocouples attached to a strip chart recorder and recordings made of the internal container temperature, external container temperature, and the chamber temperature during each experimental run.

It is evident from Figs. 4 and 5 that the rise and fall of the temperatures within the tubes and bottles lag behind the temperature outside of the containers and of the chamber. Comparing Fig. 4 for the tubes of jelly with that of Fig. 5 for the bottles of ophthalmic solution, it is evident that the temperature lags are greater for the bottle contents than for the tube contents. This can be essentially ascribed to the difference in the thermal conductance between aluminum and glass. The thermal conductance for aluminum has been reported as 0.49 cal. cm.$^{-1}$ sec.$^{-1}$ °C.$^{-1}$ while that for glass as approximately 0.0018 cal. cm.$^{-1}$ sec.$^{-1}$ ° C.$^{-1}$ (4). The above figures are representative for sterilization at 115°.

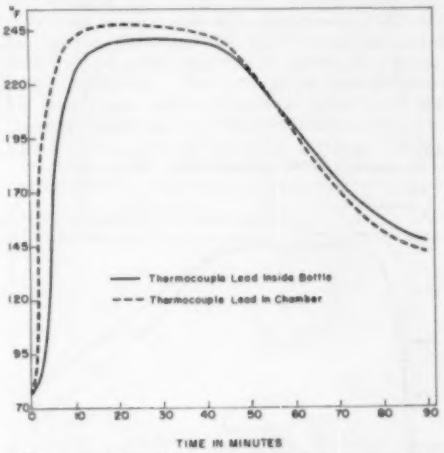

Fig. 4.—A plot of the temperature in the chamber and of the tube contents during a sterilization run in the dual-control autoclave at 115° and 25 p. s. i.

TABLE VI.– INFLUENCE OF DIFFERENT TEMPERATURES, PRESSURES (STEAM PLUS AIR), AND DURATION OF TREATMENT ON THE STERILIZATION OF CONTAMINATED JELLIES OF DIFFERENT COMPOSITIONS

Formulation	No. of Tubes Tested	Temperature, ° C.	Pressure, p. s. i.	Time, min.	Survivors, org./Gm.	No. of Tubes Sterile
Complete	3	95	15	30	0	3
	3	95	15	45	0	3
	3	105	15	30	0	3
	2	(Unheated controls)			120,000	0
Minus chlorobutanol only	4	95	15	30	<10	2
	4	95	15	45	<10	2
	4	105	15	30	0	4
	3	(Unheated controls)			350,000	0
Minus Pyribenzamine only	4	95	15	30	<10	0
	4	95	15	45	<10	3
	4	105	15	30	0	4
	3	(Unheated controls)			304,000	0
Minus chlorobutanol and Pyribenzamine	3	95	15	30	<10	0
	4	95	15	45	<10	1
	4	105	15	30	0	4
	3	(Unheated controls)			250,000	0

Inoculum—350,000 spores/Gm.

TABLE VII.—INFLUENCE OF TEMPERATURE, PRESSURE (STEAM PLUS AIR), AND DURATION ON THE STERILIZATION OF CONTAMINATED OPHTHALMIC SOLUTIONS

Formulation	No. of Samples Tested	Temperature, °C.	Pressure, p. s. i.	Time, min.	Survivors, org./Gm.	No. of Samples Sterile
Otrivin ophthalmic solution 0.05% with phenylmercuric acetate 1:100,000	4	95	10	30	0	All sterile
	4	95	15	30	0	All sterile
	4	105	15	30	0	All sterile
	4	105	20	30	0	All sterile
	4	105	25	30	0	All sterile
	4	115	15	30	0	All sterile
	4	115	25	30	0	All sterile
	4	115	30	30	0	All sterile
	3	(Unheated controls)			117,000	....
Otrivin ophthalmic solution 0.05% without preservative	4	95	10	30	0	All sterile
	4	95	15	30	0	All sterile
	4	105	15	30	0	All sterile
	4	105	20	30	0	All sterile
	4	105	25	30	0	All sterile
	4	115	15	30	0	All sterile
	4	115	25	30	0	All sterile
	4	115	30	30	0	All sterile
	3	(Unheated controls)			81,000	....
Sodium chloride solution 0.9%	4	95	10	30	0	All sterile
	4	95	15	30	0	All sterile
	3	105	15	30	0	All sterile
	4	105	20	30	0	All sterile
	4	105	25	30	0	All sterile
	4	115	15	30	0	All sterile
	4	115	25	30	0	All sterile
	5	115	30	30	0	All sterile
	3	(Unheated controls)			88,000	....

Inoculum—125,000 organisms/Gm.

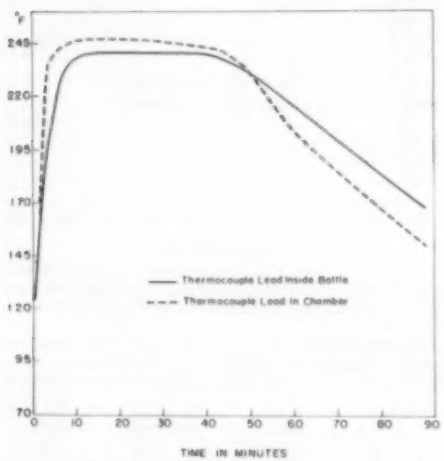

Fig. 5.—A plot of the temperature in the chamber and of the bottle contents during a sterilization run in the dual-control autoclave at 115°and 25 p. s. i.

However, similar curves are also obtained at temperatures of 95 and 105°. The lag during the cooling portion of the curve has been observed to become greater as the temperature of autoclaving is increased.

Because there appears to be a lag in the temperature within the containers during the sterilization cycle with respect to chamber temperature, a lag which undoubtedly would increase as the volume of the containers is increased, it is essential that the sterilization cycle should start when the proper temperature within the container is attained and not according to the chamber temperature.

SUMMARY AND CONCLUSIONS

It has been shown that through the use of air during the steam sterilization and subsequent cooling cycle, the bursting of collapsible aluminum tubes filled with a pharmaceutical jelly and the leakage of solution from screw-capped bottles of ophthalmic solution have been prevented. These effects have been achieved by modifying a conventional steam autoclave into a dual-control autoclave in which the pressure and temperature in the sterilizer can be controlled independently of each other.

Pharmaceutical jellies have been sterilized in the dual-control auoclave under various conditions of temperature and pressure without deleterious physical effects upon the filled collapsible tubes.

Ophthalmic solutions in screw-capped bottles have been sterilized in the dual-control autoclave under various conditions of temperature and pressure without deleterious effects upon the caps and bottles and with no leakage of solution.

Data have been presented dealing with the temperature differential of the chamber and container contents during the sterilization and subsequent cooling cycles. The thermal conductivity of the container composition plays an important role in this temperature differential.

The influence of the preservative chlorobutanol and the active constituent Pyribenzamine in Pyribenzamine anesthetic jelly, on the sterilizing capabilities of the submarginal temperatures have been investigated.

The effectiveness of submarginal temperatures on the sterilization of Otrivin ophthalmic solutions has been determined in the presence and absence of the preservative phenylmercuric acetate.

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A Note on the Synthesis of Some Alkyl and Alkylamine Esters of N,N′-Diphenylphosphorodiamidic Acid*

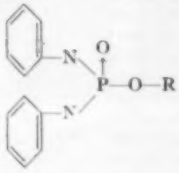
By LINDLEY A. CATES and TONY E. JONES

ESTERS OF CARBOXYLIC ACIDS in which basic groups are introduced in both acid and alcohol residues (i. e., dibutoline) and those in which the basic group is located only in the acid portion of the molecule are known to have antispasmodic activity. Since alkyl and alkylamine esters of a disubstituted phosphonous acid, N,N′-diphenylphosphorodiamidic acid, appear to possess some structural relationships to these carboxylic acid esters, a series of eight such compounds was prepared for study as possible antagonists of acetylcholine and as inhibitors of cholinesterase. These compounds, listed in Table I, were synthesized by the treatment of monochlorophosphoryl dianilide (7) with various alcohols, according to the method of Michaelis (1).

EXPERIMENTAL

Three grams of sodium metal was allowed to react with 150 cc. of the appropriate anhydrous alcohol. To the sodium alcoholate was added 8.0 Gm. of monochlorophosphoryl dianilide with an accompanying increase in temperature. The mixture was stirred for twenty minutes and the sodium chloride removed by filtration. The clear filtrate was added to 500 cc. of water with the formation of a dense, white precipitate. The precipitate was collected on filter paper and washed with water until the washings were neutral. Recrystallization from hot, dilute ethanol yielded the pure product. In the case of the *sec*-butyl derivative, the filtrate was concentrated to a syrupy mass and placed in a refrigerator overnight. The crystals that formed were collected, washed with water, and recrystallized from hot dilute ethanol. Repeated recrystallization of compound No. 8 failed to produce a completely pure product.

TABLE I.—ALKYL AND ALKYLAMINE ESTERS OF N,N′-DIPHENYLPHOSPHORODIAMIDIC ACID



No.	R	Yield, %	M. p., °C.[a]	Analysis: Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	Methyl	40	108–109	59.53	59.31	5.76	5.66
2	*n*-Propyl	61	101–103	62.05	61.18	6.59	6.62
3	iso-Propyl	60	149–150	62.05	61.18	6.59	6.95
4	*sec*-Butyl	31	111–113	63.14	63.28	6.95	7.23
5	β-Diethylaminoethyl	55	122–123	62.23	61.78	7.25	7.25
6	β-Dimethylaminoethyl	35	161–162	60.17	59.64	6.94	6.97
7	β-Dimethylamino-α-methylethyl	19	145–147	61.24	61.03	7.25	7.24
8	β-Amino-α-methylethyl	13	192–194	59.00	60.29	6.60	5.86

[a] All melting points are uncorrected.

Previously synthesized esters of N,N′-diphenylphosphorodiamidic acid are the ethyl (1), phenyl (2), *p*-chlorophenyl (3), *o*-carboxyphenyl (4), 2-chloroethyl (5), and a series of six complex esters prepared by the method of Zetzsche and Buttiker (6). No alkylamine esters have been reported until the present work.

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A Note on the Use of Citric Acid and Tartaric Acid Buffers in the Extraction of Solanaceous Alkaloids by Centrifugation*

By DANIEL P. N. TSAO

WITT, *et al.* (1), and Sciuchetti and Youngken (2) recommended McIlvain's citric acid buffer for the extraction of alkaloids from small quantities of belladonna and hyoscyamus leaves but found it less satisfactory for the complete extraction of the total alkaloid of stramonium leaf. This note describes a modified method in which a centrifugation procedure is recommended as a substitute for filtration during the extraction of solanaceous alkaloids from the plants.

EXPERIMENTAL

The experiments are divided into two parts: (*A*) comparison of the efficiency of the alkaloidal extraction when citric and tartaric acid buffers are employed according to the general method of Witt, *et al.* (1, 2), and (*B*) comparison of the above results with those obtained when a centrifugation procedure is adopted in the place of the general method described previously.

Part A.—Assays of total alkaloids of belladonna and stramonium leaf material were carried out according to the procedure of Witt, *et al.* (1, 2). Citric and tartaric acid buffers were used as menstruum. The tartaric acid buffer consisted of 75 parts of 0.1 *M* tartaric acid and 25 parts of 0.2 *M* dibasic sodium phosphate.

Part B.—The centrifugation method was carried out in the following manner: a 0.5-Gm. sample of a fine powdered leaf material was placed in a 50-ml. Erlenmeyer flask. Twenty-five ml. of the specified menstruum (citric or tartaric acid buffer) was added; the flask was stoppered and the plant material was then shaken in this solution for fourteen to sixteen hours by a mechanical shaker. Thereafter, the mixture was centrifuged and filtered into a dry glass container. Three milliliters of the filtrate was made alkaline with ammonium hydroxide T. S. and then shaken vigorously in a separatory funnel with 25 ml. of chloroform for three minutes. The remainder of the procedure followed that described by Witt, *et al.* (1, 2).

RESULTS AND DISCUSSION

Results of analyses of belladonna and stramonium leaf material are shown in Tables I and II. It was observed that the average of total alkaloids in belladonna leaves extracted by citric and tartaric acid buffers which were subsequently separated from the plant material by filtration were 0.415 ± 0.039 and 0.447 ± 0.039%, respectively. The averages of total alkaloids in the same leaf material extracted by the same two buffers but separated by centrifugation were 0.429 ± 0.045 and 0.467 ± 0.022%, respectively. In stramonium, the total alkaloids extracted by citric and tartaric acid using filtration process were 0.183 ± 0.012 and 0.201 ± 0.025%, respectively, and by the centrifugal procedure were 0.206 ± 0.022 and 0.225 ± 0.018%, respectively.

TABLE I.—ALKALOID ASSAY OF BELLADONNA LEAVES BY CITRIC ACID AND TARTARIC ACID BUFFERS, CENTRIFUGATION *vs.* FILTRATION

	—Citric Acid—		—Tartaric Acid—	
Sample	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %
1	0.375	0.390	0.400	0.417
2	0.385	0.392	0.405	0.454
3	0.390	0.421	0.420	0.458
4	0.400	0.441	0.475	0.479
5	0.457	0.500	0.480	0.492
6	0.480	...	0.500	0.500
Average	0.415	0.429	0.477	0.467
S. D.	±0.039	±0.045	±0.039	±0.022

[a] Calculated as atropine.

TABLE II.—ALKALOID ASSAY OF STRAMONIUM BY CITRIC ACID AND TARTARIC ACID BUFFERS, CENTRIFUGATION *vs.* FILTRATION

	—Citric Acid—		—Tartaric Acid—	
Sample	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %
1	0.167	0.180	0.170	0.192
2	0.175	0.187	0.185	0.203
3	0.178	0.192	0.190	0.213
4	0.185	0.192	0.195	0.215
5	0.190	0.196	0.215	0.222
6	0.204	0.202	0.250	0.226
7	...	0.219	...	0.229
8	...	0.220	...	0.240
9	...	0.221	...	0.255
10	...	0.248	...	0.266
Average	0.183	0.206	0.201	0.226
S. D.	±0.012	±0.022	±0.025	±0.018

[a] Calculated as atropine.

The tartaric acid buffer proved slightly more efficient for the extraction of solanaceous alkaloids from belladonna and stramonium leaf material than the citric acid buffer, but the increase was not significant.

The centrifugation method required about twenty minutes in comparison with three to five hours for the separation of the buffer from the plant mixture by the method of Witt, *et al.*, and Sciuchetti and Youngken (1, 2).

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